DIFFERENTIATION OF PROSTANOID RECEPTORS

BY

YIJIE DONG

THESIS SUBMITTED FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

AT THE UNIVERSITY OF EDINBURGH

MARCH 1984

DECLARATION

I Certify that the thesis has been composed by myself only. When results from other workers are quoted, the sources are given.

Yijie Dong

Note: In the text of this thesis,

PGA2 = PGA_2

PGE2 = PGE_2

PGE1 = PGE_1

PGF2a= $PGF_{2\alpha}$

PGF1a= $PGF_{1\alpha}$

PGG2 = PGG_2

PGH2 = PGH_2

PGI2 = PGI_2

PGI1 = PGI_1

TxA2 = TxA_2

CONTENTS

ABSTRACT

This investigation concerns the definition and differentiation of prostanoid receptors and the structure-activity relationships of prostanoid analogues in relation to smooth muscle responses and platelet aggregation.

In initial studies on the bullock iris sphincter preparation it was observed that the potent prostacyclin mimetic ZK 36374 partially contracted the preparation and opposed the contractile action of PGE_2, but not that of 11,9-epoxymethano PGH_2. These effects were explained in terms of a partial agonist action of ZK 36374 on the PGE_2 receptor. By observing this activity of ZK 36374 and the order of potency of other PGE_2 analogues on several different preparations, it was demonstrated that PGE_2 receptors can be divided into two subtypes. Subtype I is associated with stimulant activity on smooth muscle and the 16-p-chlorophenoxy-17,18,19,20-tetranor analogue of PGE_2 (ICI 80205) is a highly potent agonist; subtype II is associated with inhibitory activity (relaxation of cat trachea) and ICI 80205 and ZK 36374 have very low activity.

The bullock iris sphincter was also shown to contain a TxA_2 receptor system on which the endoperoxide analogue EP 011 was a particularly potent agonist. Two prostanoid analogues with

semicarbazone ω-chains, EP 045 and EP 116, were shown to be specific thromboxane receptor antagonists of high affinity. Activities of prostanoid analogues on rat platelets were also examined. CTA_2, PTA_2 and 15-oxo EP 011 gave a shape change only, while EP 011, 11,9-epoxymethano PGH_2 and 9,11-azo PGH_2 aggregated the platelets. PGE_2 potentiated the aggregatory action but not the shape change. The properties of the receptors in these different preparations are discussed.

On $PGF_{2\alpha}$ receptor sites ICI 81008 was found to be a selective agonist. We have also examined other compounds related to ICI 81008 which may be partial agonists, but desensitization complicates the analysis.

PGI_2 receptors mediating excitatory effects in the rabbit iris sphincter were shown to have properties similar to those of receptors mediating inhibitory effects in platelets.

Finally, we have found that calcium antagonists potentiate the contractile effect of prostanoids in the rat anococcygeus muscle. The possible mechanisms involved have been discussed.

Publications

Effects of Prostaglandins and Thromboxane Analogues on Bullock and Dog Iris Sphincter Preparation (Dong Y.J. & Jones R.L., 1982 Br.J.Pharmac. 74, 149)

Differentiation of PGE2 Receptors: Activity of the Carbacyclin ZK 36374 (Dong Y.J. & Jones R.L., 1982 In: Vth. International Conference of Prostaglandins, Florence, Italy, p676)

Actions of Prostanoids on the Rat Anococcygeus Muscle: Enhancement by Verapamil (Dong Y.J. & Jones R.L., 1983 In: Proceedings of the British Pharmacological Society, 6-8th April, Cambridge, p119)

Structures of Prostanoids

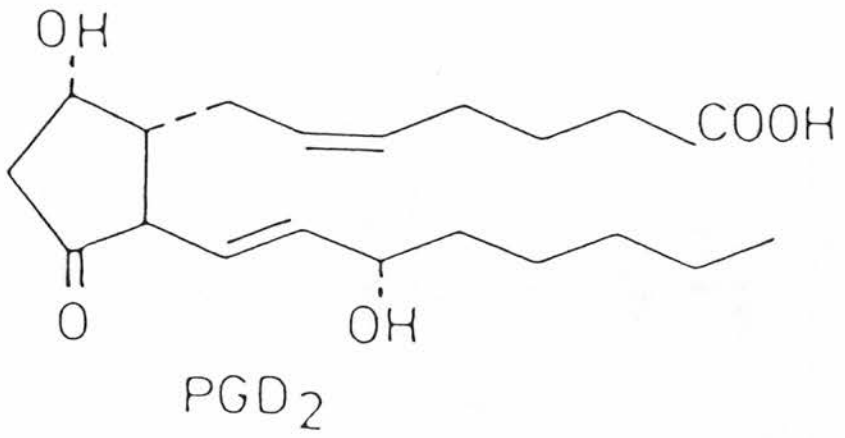


Figure S.1 Chemical structure of PGD_2

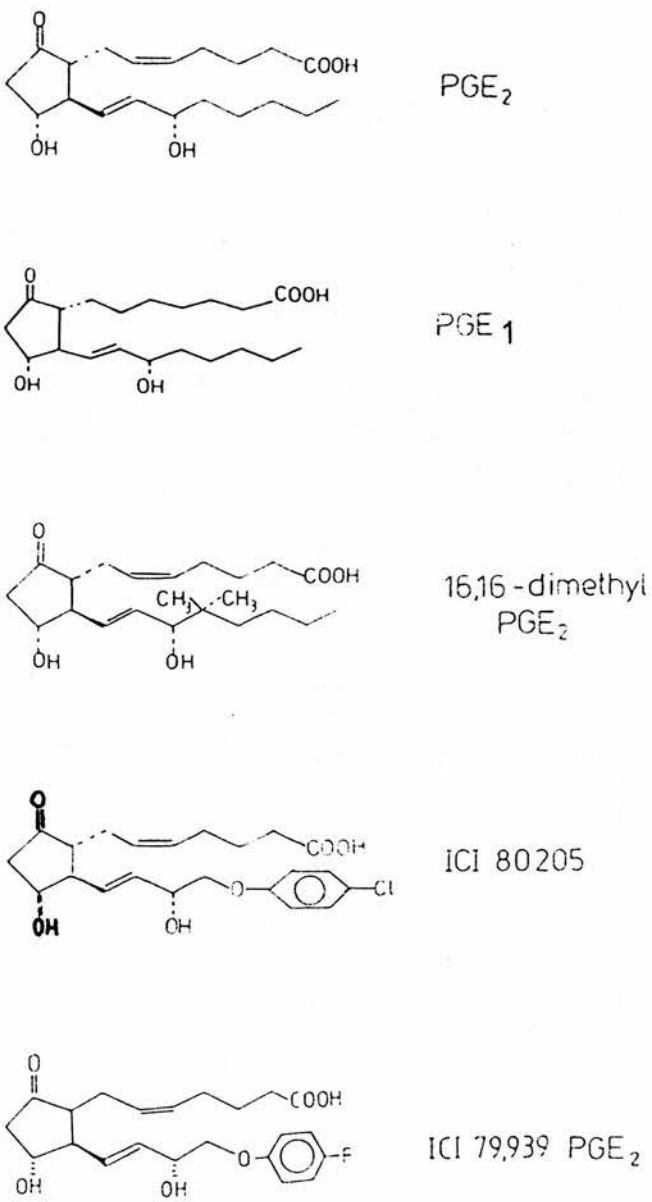
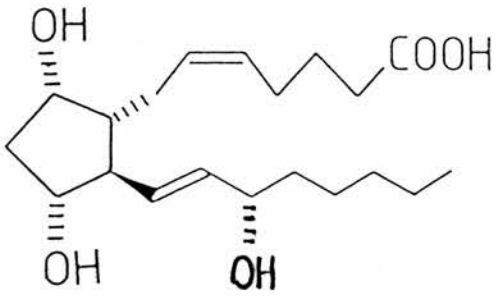
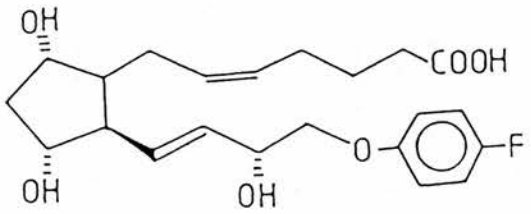


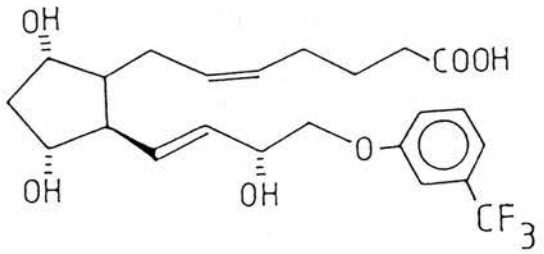
Figure S.2 Chemical structures of PGE_2 analogues



$PGF_{2\alpha}$



ICI 79939 $PGF_{2\alpha}$



ICI 81008

Figure S.3 Chemical structures of $PGF_{2\alpha}$ analogues

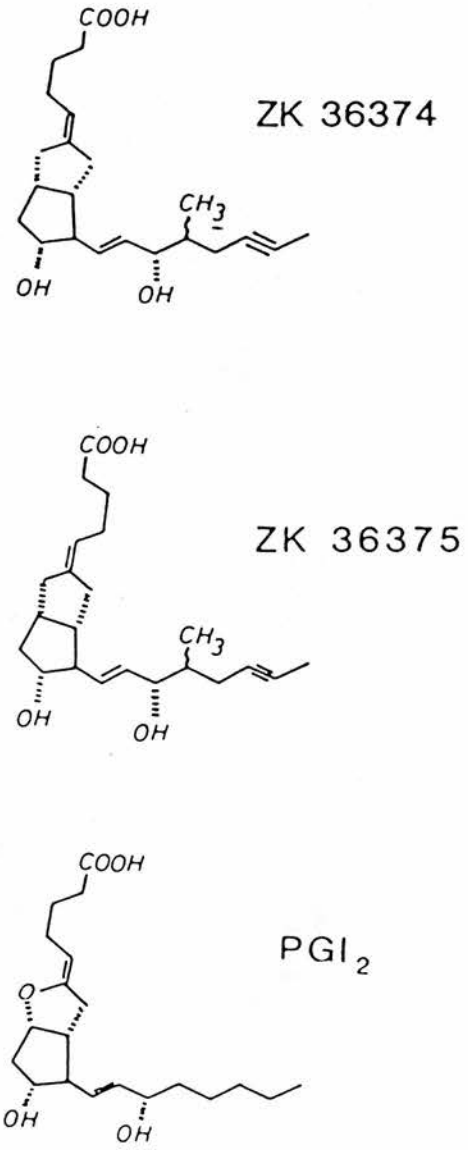


Figure S.4 Chemical structures of PGI_2 analogues

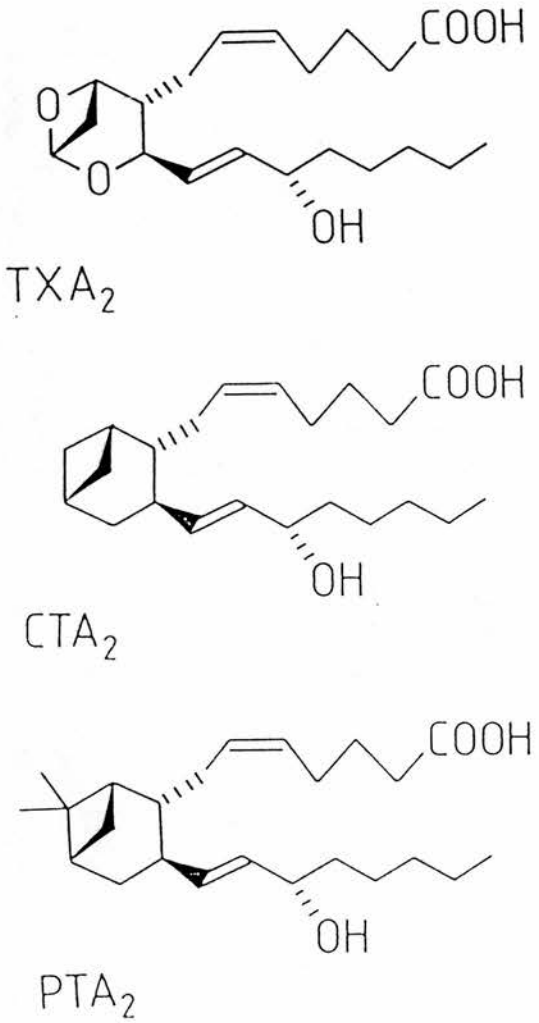


Figure S.5 Chemical structures of TxA_2 analogues

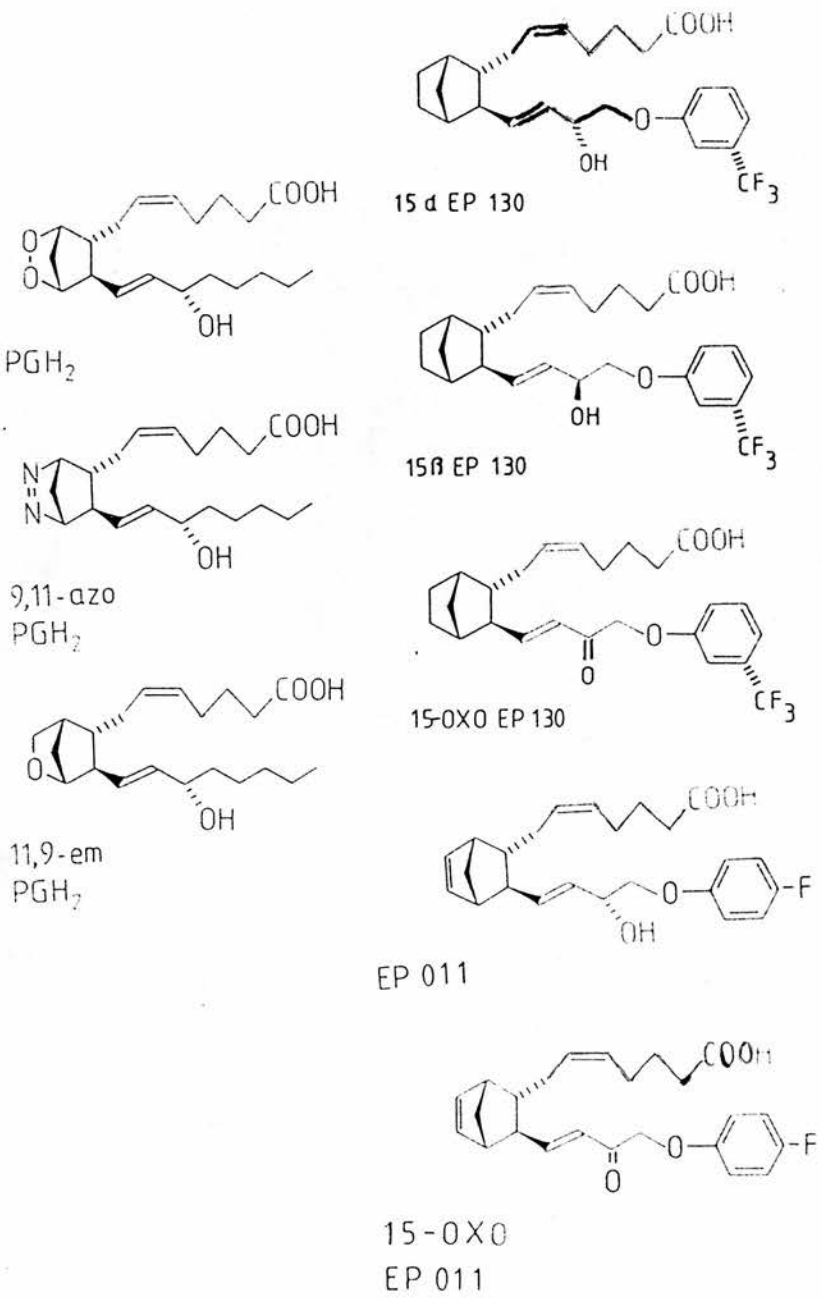


Figure S.6 Chemical structures of PGH_2 analogues

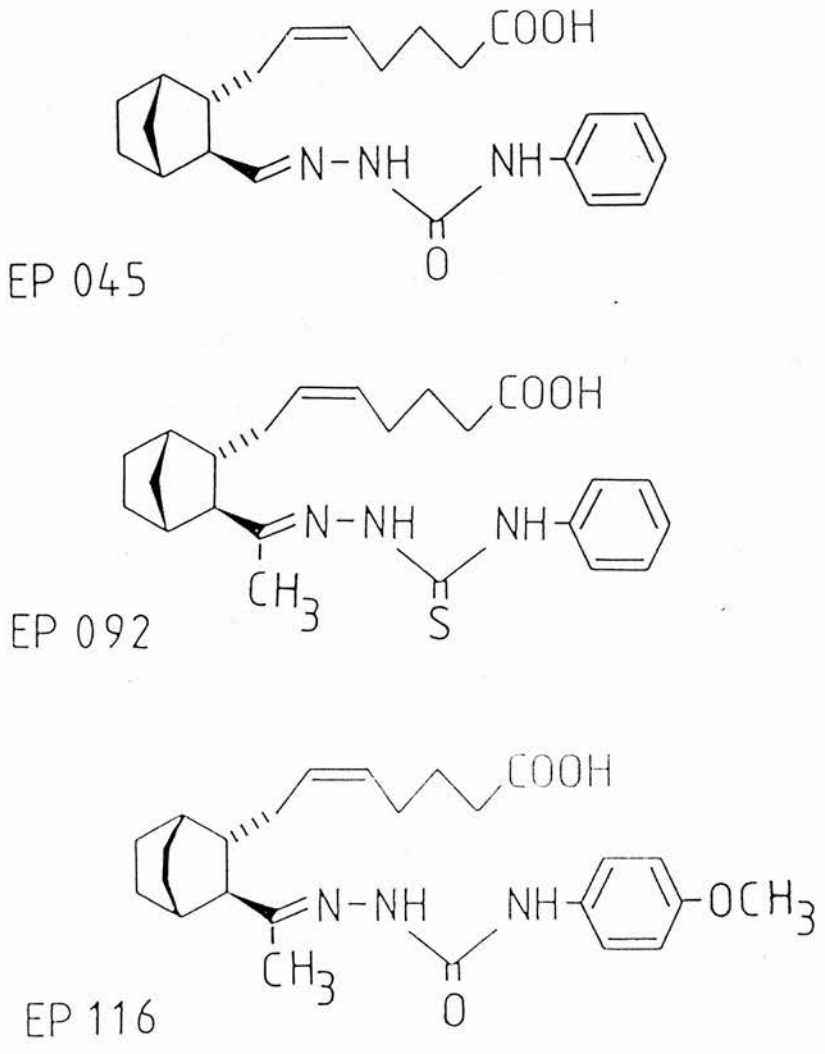


Figure S.7 Chemical structures of TxA_2 receptor antagonists

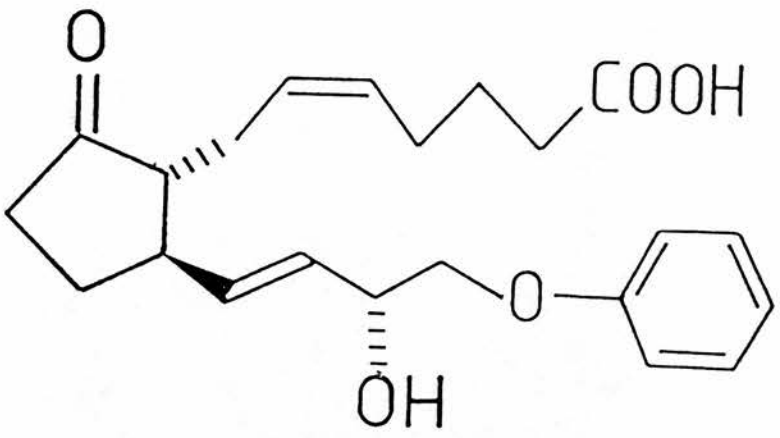


Figure S.8 Chemical structure of M/B 28767

Acknowledgements

It renders me great pleasure to record my gratitude to Dr. R.L.Jones for his excellent supervision of my work, for his encouragement, help and friendship towards me throughout my study.

I am indebted to all the staff members in the Department of Pharmacology headed by Prof. B. Ginsborg for their help, kindness and the friendly atmosphere.

I wish to thank Dr. T.Muir, Dr. R.M.Wadsworth and Dr. R.H.Michel for their advice and help.

The work in this thesis was supported by a grant from the Chinese Government and a Studentship from the University of Edinburgh.

Sources of Drugs and Chemicals

The drugs listed below were gifts, and were used in the study in Section 5:

Trifluoperazine (Smith Kline & French Laboratories Ltd.)

Trifluoperazine sulfoxide (Smith Kline & French Laboratories Ltd.)

Trypsin (C P Laboratories Limited)

Cis-Flupenthixol Dihydrochloride (H Lundbeck & Co.)

Trans-Flupenthixol Dihydrochloride (H Lundbeck & Co.)

Pimozide (Janssen Pharmaceutica)

Penfluridol (Janssen Pharmaceutica)

Amitriptyline (Merck Sharp & Dohme)

Verapamil (Knoll AG)

D 600 (Knoll AG)

General Introduction

Reference citation: PP 355 - 370

GENERAL INTRODUCTION

Nearly fifty years ago an endogenous substance with vasodepressor and smooth muscle stimulating activity was first described in accessory sex glands of the ram and seminal fluid from man independently by Goldblatt (1933) and von Euler (1934). von Euler first referred to this new class of pharmacological agent as prostaglandin . About 30 years elapsed between these original observations and full appreciation by the scientific community of the implications for research. In the 1960's a number of 20-carbon, unsaturated, hydroxy and keto substituted fatty acids with prostaglandin-like activity were isolated in crystalline form from sheep prostate glands and human seminal plasma (Bergstrom & Sjovall, 1960; Bergstrom, Ryhage, Samuelsson & Sjovall, 1963; Samuelsson 1963). This signalled the start of intensive chemical effort directed towards the total synthesis of the natural prostaglandins and related analogues.

In the following years, two independent groups (Bergstrom, Danielson & Samuelsson, 1964; von Dorp, Beerthuis, Nugteren & Vonheman, 1964) demonstrated that prostaglandins are biosynthesized from polyunsaturated fatty acid. These acid include dihomo γ-linolenic acid (C20:3ω6), arachidonic acids (C20:4ω6), and eicosapentaenoic acid (C20:5ω3), which give rise to the mono-, di- , or trienoic prostaglandins respectively. Arachidonic acid, the precursor of all di -enoic prostaglandins, is the most common fatty acid

present in cellular phospholipids and can be obtained directly from the diet or by desaturation and chain elongation from dietary linoleic acid (C18:2W6). Arachidonic acid is liberated from membrane phospholipids by the action of phospholipases, activated by stimuli of various kinds, mechanical, thermal, chemical, immunological and others (Vonkeman & von Dorp, 1968; Flower & Blackwell, 1976).

Since 1973, there have been important discoveries about the nature of the intermediates in arachidonic acid metabolism. These substances include the prostaglandin endoperoxides (PGG2 and PGH2) (Hamberg & Samuelsson, 1973; Willis & Kuhn, 1974; Willis, Vane, Kuhn, Scott & Petrin, 1974; Hamberg, Svensson & Samuelsson, 1974; Hamberg, Svensson, Wakabayashi & Samuelsson, 1974), thromboxane A2 (TxA2) (Hamberg, Svensson & Samuelsson, 1975) and prostacyclin (PGI2) (Moncada, Gryglewski, Bunting & Vane, 1976a; Moncada, Gryglewski, Bunting & Vane, 1976b). Since the structural similarity between prostaglandins and thromboxanes is evident, and thromboxanes are derived from prostaglandins, in this thesis the word "prostaglandin" (PG) or "prostanoid" will be used as a general term to describe prostaglandins, thromboxanes and the numerous metabolites and analogues structurally related to the parent compounds.

Arachidonic acid can also be metabolized by a 5-lipoxygenase to form a family of compounds containing a conjugated triene structure, named leukotrienes (Murphy, Hammarstrom & Samuelsson, 1979). Figure 1 shows the two pathways in arachidonic acid metabolism. The two peptido-lipids LTC4 and

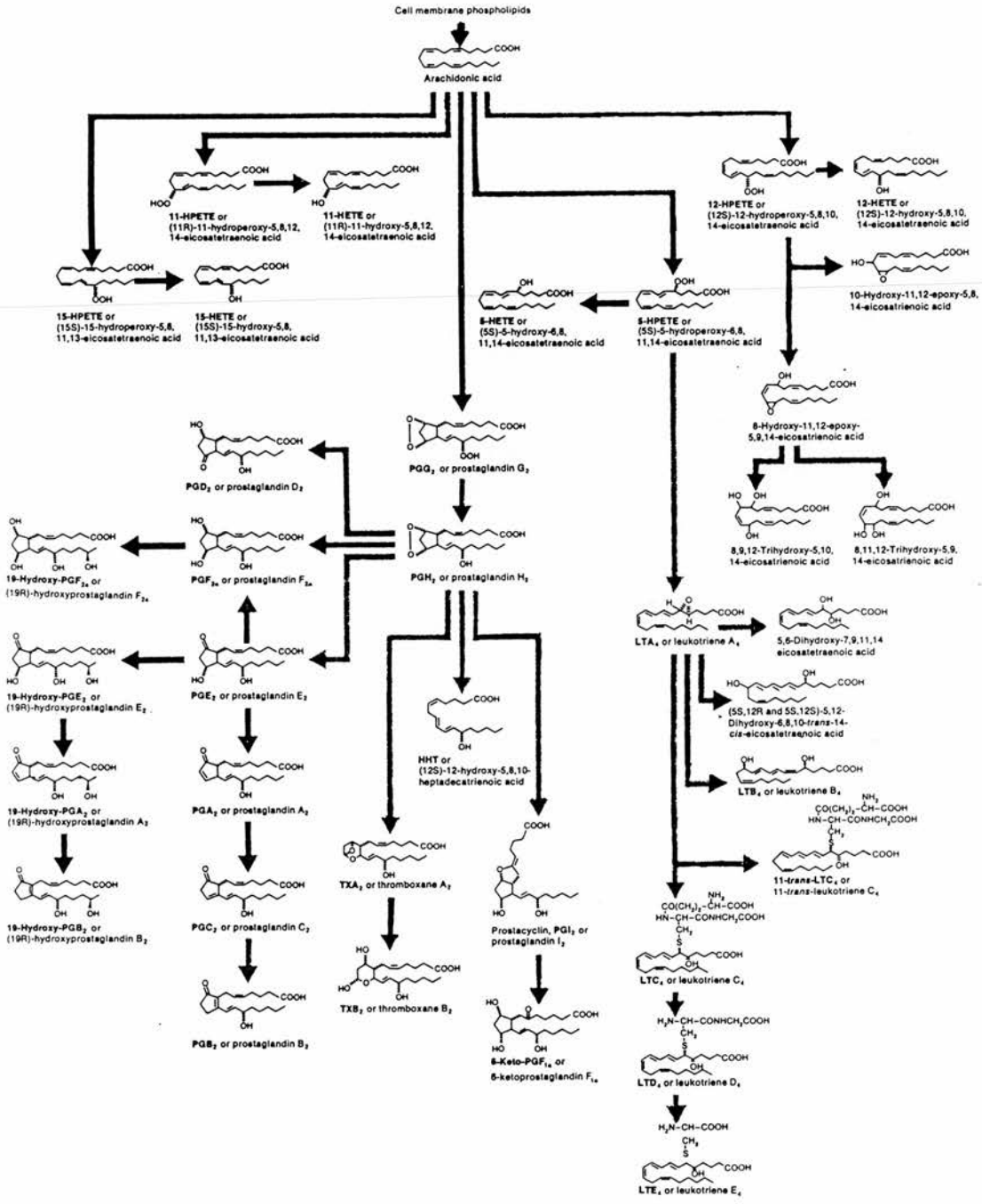


Figure 1 Biosynthesis of prostaglandins, thromboxanes and leukotrienes from arachidonic acid.

LTD4 are responsible for much of the smooth muscle stimulating activity of the slow-reacting substance (SRS-A) liberated in anaphylaxis. The dihydroxy acid LTB4 on the other hand has potent leucocyte-attracting properties (Hammarstrom, 1983).

Prostanoids have two significant features: their widespread occurrence and broad spectrum of biological activity. They can be biosynthesized by every type of animal cell with the exception of the red blood cell, and nearly every cell type can respond in some way to one or more of them. The number of reported effects of the prostaglandins and related substances is staggering and seems to encompass most biological functions in the body. They include effects on all kinds of smooth muscle, on secretory functions, on resorption of electrolytes and water, on neurotransmission, platelet aggregability, induction of fever, chemotaxis and other inflammatory responses, and a variety of endocrine and metabolic effects.

Prostanoids possess such a wide range of biological activities that it seems impossible to make useful generalizations on their mode of action. It was proposed that prostaglandins acted as calcium ionophores to transport calcium directly (Gerrard, Townsend, Stoddart, Witkop & White, 1977; Gerrard, Peterson, Townsend & White, 1976). Ionophores are molecules with backbones of diverse structures containing strategically placed oxygen atoms (Pressman, 1976). The backbone can assume conformations which focus these oxygen atoms about a ring into which an

ion of suitable size may fit. It was suggested that the oxygens of two or possibly more prostaglandins could form a hydrophilic cavity around a dehydrated calcium ion (Eagling, Lovell & Pickles, 1976; Reed & Knapp, 1978). It was shown that prostaglandins could transport calcium from a water phase into diethyl ether at physiological pH (Gerrard, Peterson, Townsend & White, 1976) and that a higher affinity was shown for calcium ions in this respect than for magnesium, potassium and sodium ions (Gerrard, White & Peterson, 1978). On the other hand, prostaglandins may displace calcium ions from superficial membrane-binding sites (White, Rao & Gerrard, 1974). However, there is considerable evidence to suggest that the versatile effects of prostaglandins are mediated by interacting with specific receptors. Investigations have shown that the actions of prostaglandins on a number of isolated organs were not modified by any of the following drugs: alpha- and beta-adrenergic blocking agents, antihistamines, anticholinergic drugs, serotonin antagonists, tubocurarine, and hexamethonium; prostanoids are active at very low concentrations; they show a high degree of chemical specificity, i.e. small changes in chemical structure have profound effects on potency and there are marked differences in potency between different isomers of the same compound. These properties suggest the existence of specific recognition sites or receptors, Indeed, many studies have revealed the existence of specific prostanoid-binding sites in the membranes of a number of cell types.

The first direct evidence for a prostaglandin receptor based

on actual competition studies between labelled and unlabelled prostaglandins was obtained using the intact lipocyte (Kuehl & Humes, 1972). Since then prostaglandin binding sites have been described in a number of tissues, e.g. corpus luteum (Powell, Hammarstrom & Samuelsson, 1973; Rao, 1976, 1977; Lin & Rao, 1978; Mattioli, Galeati & Seren, 1983; Wright, Pang & Berman, 1980; Wright, Luborsky-Moore & Berman, 1979), adipocytes (Kuehl & Humes, 1972; Gorman & Miller, 1979), thyroid (Moore & Wolff, 1973), adrenal (Dazod, Morera, Bertrand & Saez, 1974; Kapalis & Powell, 1981a, 1981b), prostate (Cavanaugh & Farmworth, 1977), ovary (Rotondi & Knazek, 1982), oviduct (Riehl & Harper, 1981), uterus (Johnson, Jessup & Ramwell, 1974; Kimball, Kirton, Spilman & Wyngarden, 1975; Kimball & Wyngarden, 1975) platelets (McDonald & Stuart, 1974; Schilliger & Prior, 1980; Shafer, Coper, O'Hara & Handin, 1979; Armstrong, Jones & Wilson, 1983; Siegl, Smith & Silver, 1980), lymphocytes (Melman, Weinstein, Sheaver & Bourne, 1974), thymocytes (Schaumberg, 1973), macrophages (Opmeer, Adolfs & Bonta, 1983), blood vessels (Rucker & Schror, 1983), gut (Tepperman & Soper, 1981a, 1981b, 1983; Miller & Magee, 1973; Kantor, Tao & Kiefer, 1974), liver (Okamici & Terayama, 1977; Smigel & Fleischer, 1974), brain (Comaty, Ehrenpreis & Greenberg, 1976), lung (MacDermot, Barnes, Waddell, Dollery & Blair, 1981).

However, in most cases it has yet to be established whether these are the receptors mediating the biological actions of prostanoids or sites related to their transport or enzymatic inactivation, since according to the terminology by Paton

(1961) binding sites in which there is a high degree of target specificity may be classified as receptors or acceptors. In general a receptor is considered to be a component of the cell which upon interaction with a specific stimulator, generally from an external source, initiates a series of biochemical or/and physiological events characteristic of the cell.

Different prostanoids often produce opposite effects on the same cell type. For instance, TxA2 activates platelets to aggregate, while PGI2 inhibits platelet aggregation; some types of smooth muscle are contracted by PGF2a but relaxed by PGE2. Furthermore, different cell types respond to different prostanoids. Tachphylaxis to one PG may leave the response to another unchanged. Substances such as SC-19220(P333), 7-oxa-13-prostynoic acid, or polyphloretin phosphate block contractions produced by some PGs but do not block the relaxations produced by the same PGs on other smooth muscle preparations. Some antagonists also appear to be more effective in antagonizing responses to one PG than another even though the actions of the PGs are the same type. From the qualitative and quantitative differences in the biological properties of the various natural prostaglandin families, it is clear that several different prostaglandin receptors must exist. In 1967, Pickles suggested that there were more than two types of prostaglandin receptors (Pickles, 1967). In late 1970's Jones claimed that there were five such receptors (PGD, PGE, PGF, PGI and TxA receptors) (Horton, 1979). This idea is supported by the work of Kennedy, Coleman, Humphrey, Levy and Lumley (1982).

The differentiation of prostanoid receptors is of more than academic interest, since the number of different types and their distribution in different tissues will largely determine the degree of selectivity obtainable with sythetic compounds that mimic or block the effects of the natural compounds.

Generally, the approaches used to characterize receptor-mediated process include the following:

(1) Measuring the rank order of potency of agonists.

(2) Desensitization using high concentrations of agonists.

(3) Use of specific antagonists.

(4) Ligand binding studies.

We have used procedures (1), (2) and (3) to define and differentiate prostanoid receptors by observing the changes in mechanical responses in isolated smooth muscle preparations, and shape change, aggregation and granular secretion in platelet suspensions.

Part One

Reference citations: pp 355 - 370

Section One

Actions of Prostanoids on Eyes

of Bullock, Dog, Cat, Horse and Rabbit

Reference citations : PP 355 - 370

INTRODUCTION

Interest in the actions of prostaglandins on the eye stems from the identification of E- and F-type prostaglandins as components of lipid extracts (irin) from sheep (Anggard & Samuelsson, 1964), rabbit and cat (Ambache, Brummer, Rose & Whiting, 1966) and bullock (Posner, 1970) irides. von Dorp, Jouvenaz and Struijk (1967) also showed that pig iris could convert all- cis-8,11,14-eicosatrienoic acid into prostaglandins E1 and F1a.

Several workers have made pharmacological comparisons of prostaglandins on isolated iris smooth muscle. The dog and cat sphincter muscles are of particular interest since PGF2a produces a contractile effect at very low concentrations and is much more active than PGE2 and stable TxA2-like agents such as 11.9-epoxymethano PGH2 (van Alphen & Angel, 1975; Coleman, Humphrey, Kennedy, Levy & Lumley, 1981). In contrast, the bullock iris sphincter is more responsive to PGE2 than PGF2a (Posner, 1973). We decided to characterize further the PGE-sensitive system in the bovine iris sphincter, with a view to comparing it with other smooth muscle preparations responsive to PGE2 and related analogues.

METHODS

Bullock and horse eyes were enucleated just after slaughter, placed in ice-cold sugar-salt solution (KCl 0.35, NaCl 6.95, $CaCl_2$ 0.24, $MgSO_4 \cdot 7H_2O$ 0.29, KH_2PO_4 0.16, $NaHCO_3$ 1.25, dextrose 1.0, sucrose 17 and EDTA 0.01 g/l) (Crawford, van Alphen, Cook & Lands, 1978), and used within 12 h. Two strips of the sphincter muscle, about 10 mm long and 2.5 mm wide, were dissected from the upper and lower margins of the bullock iris and from the right and left margins of the horse iris, respectively. Tension 40-60 mg was chosen for the bullock iris sphincter and 500 mg for the horse iris sphincter.

Dog and cat eyes were removed during pentobarbitone anaesthesia and rabbit eyes were enucleated immediately after they had been killed. A loop of the sphincter muscle was dissected by a circular cut 2 mm from the pupillary margin, and tension 40-60 mg was chosen.

All the preparations were suspended in 10 ml of the bathing solution containing indomethacin (1 μM), aerated with 95% O_2 and 5% CO_2 and kept at 37°C. The effect of drugs on muscle tension was measured isometrically with a Grass force displacement transducer (FT 03C) and recorded on a Grass Polygraph (Model 7C). Each preparation was allowed 30-60 min to stabilize.

Prostaglandins were dissolved in 0.9% w/v NaCl solution (saline) with addition of small amounts of sodium

bicarbonate when necessary. PGI2 sodium salt (Schering) was dissolved in 0.05 M Tris HCl, pH 9.0 to give a 1 mg/ml solution. Serial dilutions of the PGI2 stock solution were made with saline, kept on ice and used for one cumulative dose sequence only.

RESULTS

Bullock Iris Sphincter Muscle

Relative Potencies of Prostanoids

As a routine procedure the bathing fluid contained indomethacin; this ensured a stable basal tension over a period of at least 5 h. The preparation is sensitive to both 11.9-epoxymethano PGH2, a thromboxane analogue, and PGE2, each producing a dose-dependent contraction. A number of analogues of both TxA2 and PGE2 were tested. Doses were added cumulatively to the organ bath. Figure E.1 illustrates a typical trace of responses of the bullock iris sphincter preparation to cumulative doses of a prostanoid. Responses to most prostanoids reached a steady level after 3-10 min and were sustained for at least 10 min. Following wash-out of the organ bath, tension of the muscle gradually returned to the resting level. The interval between two sequences was about one hour. The response was taken as the absolute increase in muscle tension above the resting tension. Log concentration-response curves were plotted.

PGE2 was used as the standard agonist for PGE2 sensitive sites and 11.9-epoxymethano PGH2 for TxA2 sensitive sites. Preparations were reproducibly responsive to PGE2 and 11.9-epoxymethano PGH2. Threshold responses to PGE2 (tension change of about 20 mg) were seen with concentrations of 0.2-0.4 ng/ml and 50% maximum responses (150-400 mg) with

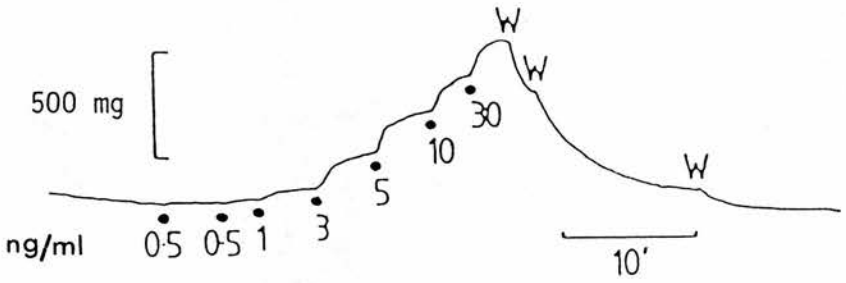


Figure E.1 Bullock iris sphincter muscle: responses to cumulative doses of 11,9-epoxymethano PGH_2. The organ-bath contained sugar-salt solution with indomethacin 10^{-6} M. W = Wash.

1-6 ng/ml. Representative log concentration-response curves are shown in Figure E.2. Log concentration-effect curves for all the analogues listed in Table E.1 were approximately parallel to that of PGE2. Equipotent molar ratios (EPMR) (Table E.1) were derived from the molar concentration of PGE2 giving a response 50% of its own maximum and the molar concentration of the test compound producing an equivalent response.

On eight preparations the 11.9-epoxymethano analogue of PGH2 (U-46619) showed similar contractile potency to PGE2. but its log concentration-effect curve was always slightly steeper and its maximum response greater (5-25%). At the 50% maximum response level for PGE2. 11.9-epoxymethano PGH2 was 1.25 (range 0.50-2.7, n=8) times less active than PGE2.

Addition of PGE1, PGE2 or 11.9-epoxymethano PGH2 to a preparation partially contracted with carbachol resulted in a further increase in tension: there was no evidence for a prostaglandin-mediated relaxant effect on this preparation. Adrenaline (200 ng/ml) completely inhibited the contractile action of carbachol.

Partial Agonist Action of ZK 36374

ZK 36374, a compound known to have potent PGI2-like activity was found to be considerably more active than PGI2. In addition it produced a lower maximum response than either PGE2 or 11.9-epoxymethano PGH2 (Figure E.2a). This was considered of interest and further experiments were conducted to determine whether ZK 36374 was a partial

Table E.1 Potencies of prostanoid analogues on the bullock isolated iris sphincter preparation

Compound	Equipotent molar ratio (PGE_2 = 1.0)				
	individual value				mean ± s.e.
(±) ICI 80205	0.12	0.16	0.17	0.17	0.16 ± 0.012 (n=4)
16,16-dimethyl PGE_2	0.19	0.23	0.28	0.29	
	0.37	0.42	0.62	0.70	
	0.86				0.44 ± 0.078 (n=9)
PGE_1	1.7	5.3	9.3		
(±) 11-deoxy PGE_1	37	47			
PGD_2	480	690	800	2000	
$PGF_{2\alpha}$	19	19	21	56	44 ± 12 (n=6)
	63	86			
(±) ICI 81008	(640)	(650)	1000		
PGI_2	40	87	100	120	135 ± 26 (n=7)
	170	180	250		
6-β PGI_1	18	40	48	50	51 ± 13 (n=5)
	99				

Individual values are the result of a comparison with PGE_2 on a single preparation. With ICI 81008, the full concentration-response relationship was not established in two experiments and the corresponding equipotent molar ratios in parentheses relate to responses at the 20% maximum response level. PGI_1 and PGI_2 produced 77 ± s.e. 0.39%(n=7) and 76 ± s.e. 2.8%(n=7) of the maximum response obtained with PGE_2, respectively.

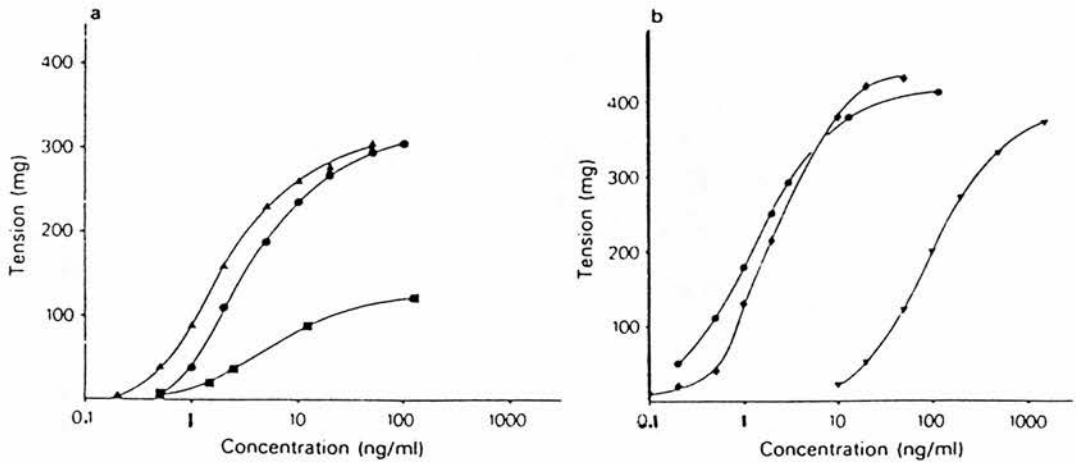


Figure E.2 Bullock iris sphincter muscle preparations: cumulative concentration-response relationships for (a) PGE_2 (●), 16,16-dimethyl PGE_2 (▲) and ZK 36374 (■); (b) PGE_2 (●), 11,9-epoxymethano PGH_2 (◆) and $PGF_{2\alpha}$ (▼).

agonist on either the PGE- or TxA-sensitive systems.

On eight preparations the maximum response to ZK 36374 ranged between 33 and 75% (mean=47%) of the maximum obtained with PGE2. The interaction of ZK 36374 with other agonists, PGE2, 16,16-dimethyl PGE2, 11,9-epoxymethano PGH2, PGF2a, PGI2 and carbachol was studied. The procedure involved the establishment of a cumulative dose-response curve to the full agonist followed by a second cumulative curve in the presence of a fixed concentration of ZK 36374 (0.1 or 0.3 µg/ml). Typical results are shown in Figure E.3. The contractile action of both 11,9-epoxymethano PGH2 and carbachol was apparently additive with that of ZK 36374. However, ZK 36374 opposed the contractile action of PGE2, 16.16-dimethyl PGE2, PGF2a and PGI2. This activity of ZK 36374 is consistent with a partial agonist action at the PGE-sensitive system.

It is noteworthy that PGI2 sodium salt was dissolved in 0.05 M Tris HCl, pH 9.0. and serial dilutions were made with saline immediately before use. At first we made a 100 µg/ml stock solution using the Tris HCl buffer. The interaction of PGI2 with ZK 36374 appeared additive. Effects of the solvent on the response to ZK 36374 were examined. It was shown that Tris HCl caused a concentration-dependent potentiation of the contractile action of ZK 36374 and PGI2, when the final concentration of Tris HCl was higher than 0.2 µM. Therefore, a 1 mg/ml stock solution of PGI2 was made. The solvent potentiation effect was attenuated and ZK 36374 opposed the action of PGI2.

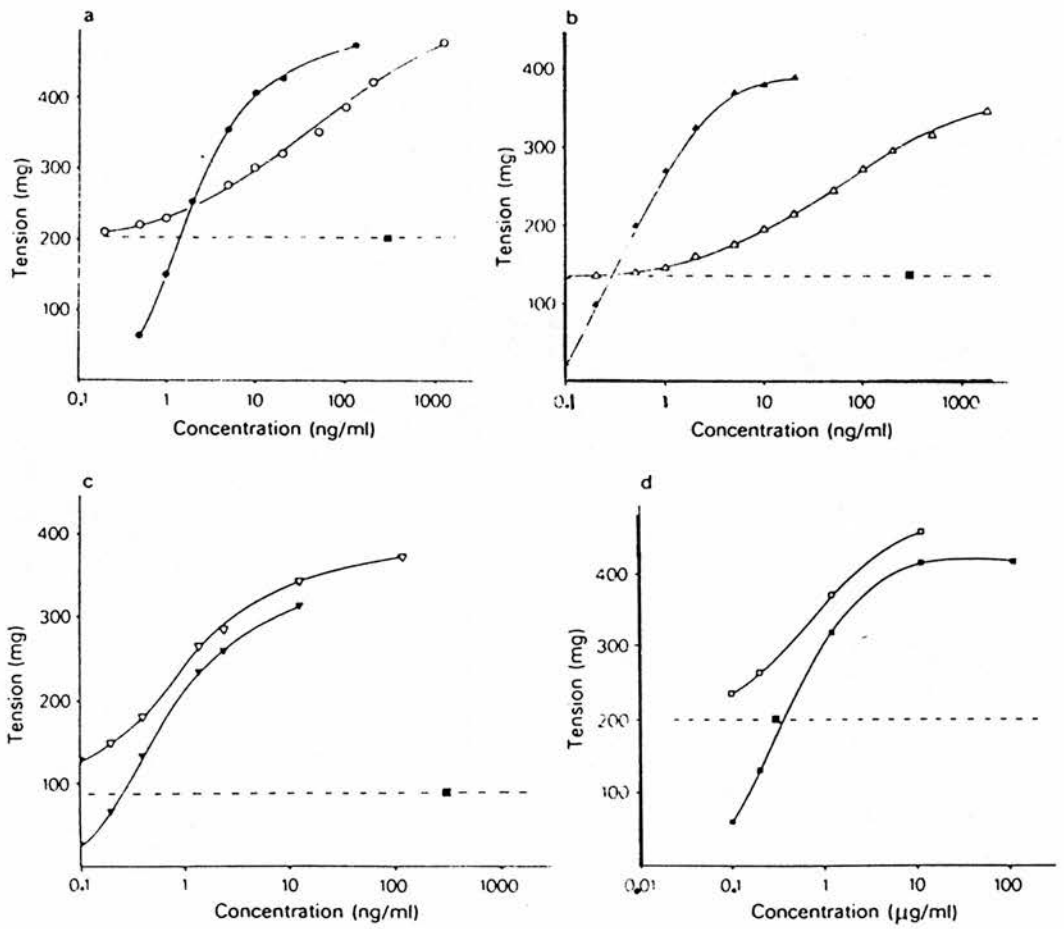


Figure E.3 Bullock iris sphincter muscle: interaction of ZK 36374 with (a) PGE_2, (b) 16,16-dimethyl PGE_2, (c) 11,9-epoxymethano PGH_2 and (d) carbachol. In each instance a cumulative concentration-response relationship was first established to the full agonist alone (solid symbols), followed by a cumulative relationship (open symbols) in the presence of a fixed concentration of 0.3 µg/ml ZK 36374 (■)

Estimates of the affinity constant of ZK 36374 were made using the log concentration-effect curves for PGE2 and ZK 36374 obtained on the same preparation. It was assumed that PGE2 had a high efficacy and that for submaximal responses receptor occupancy was both small and directly proportional to its concentration. The concentration of PGE2 which gave a response equal to the ZK 36374 maximum response (100% occupancy by ZK 36374) was found first. The response which corresponded to half this concentration of PGE2 was then found and finally the concentration of ZK 36374 which gave a response identical in size to it was read off (Figure E.4). The reciprocal of the ZK 36374 concentration (corresponding to 50% occupancy) was taken as the affinity constant. From six experiments the mean affinity constant was calculated to be 1.03 ($\pm$ 0.17 s.e.mean) x 10^8 M^{-1}.

Thromboxane-like Analogues

Several compounds with thromboxane-like activity on other smooth muscle preparations were tested. These were 11,9-epoxymethano PGH2, EP 011, 15-oxo EP 011, 9,11-azo PGH2, 9,11-ethano PGH2, CTA2 and PTA2. Typical concentration-response curves are shown in Figure E.5. EP 011, 15-oxo EP 011, CTA2 and PTA2 were slow both in on-set and off-set on the bullock iris sphincter preparation. Table E.2 summarizes the relative potencies of these agents on the preparation. 11,9-Epoxymethano PGH2 was taken as the standard agonist. For a full agonist equipotent molar ratios were determined by calculating the quotient between the molar concentration of 11,9-epoxymethano PGH2

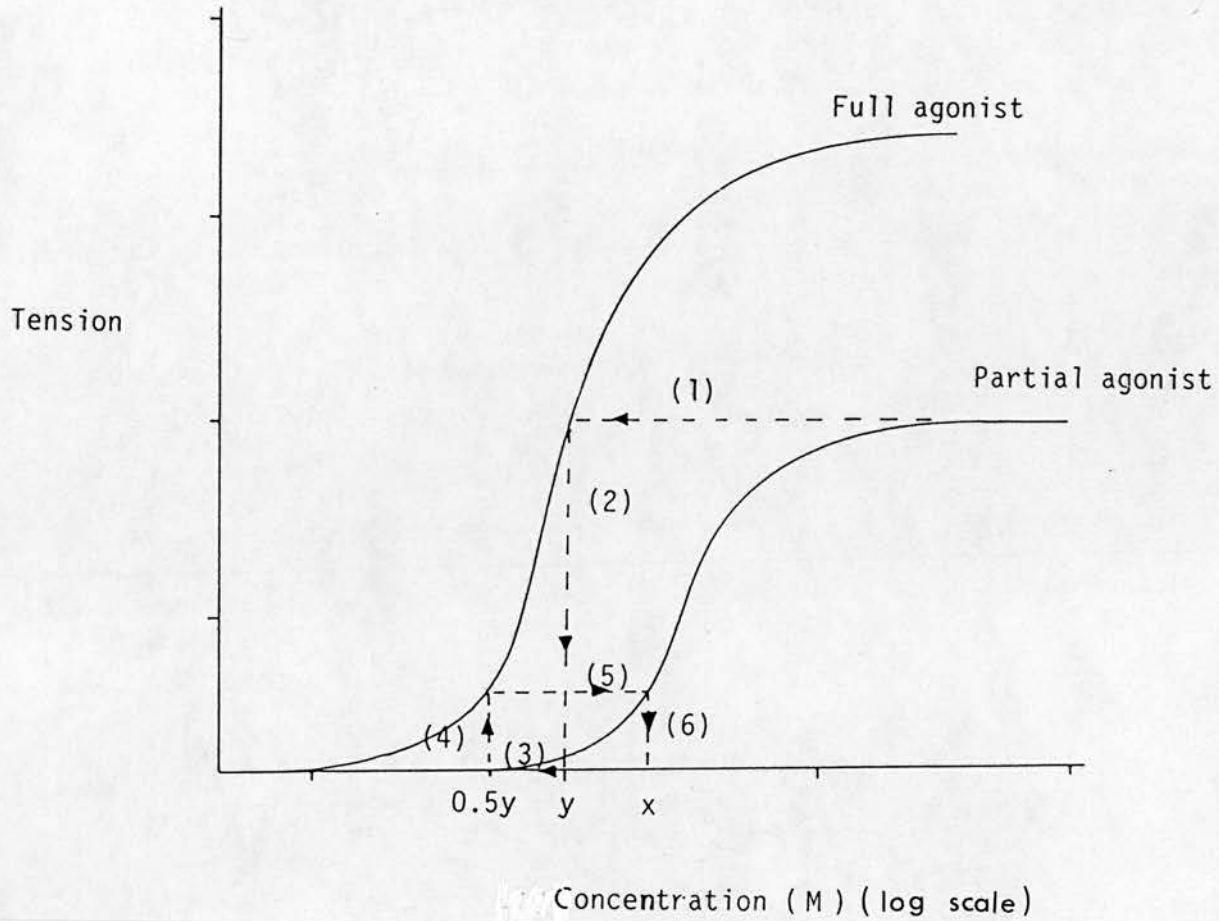


Figure E. 4 Schematic diagram: calculation of the affinity constant for a partial agonist.

y = molar concentration of the full agonist producing a response equal to the maximum response inducible by the partial agonist [(1) + (2)] i.e. response induced by the partial agonist when occupying 100% of the receptors.

0.5 y = molar concentration of the full agonist producing the response [(3) + (4)] resulting from the occupation of 50% of the receptors occupied by concentration y.

x = molar concentration of the partial agonist producing a response equal to that induced [(5)] by 0.5 y and is thus the concentration of the partial agonist required for occupation of 50% of the total population of the receptors.

The affinity constant of the partial agonist will thus be given by $\frac{1}{x}$.

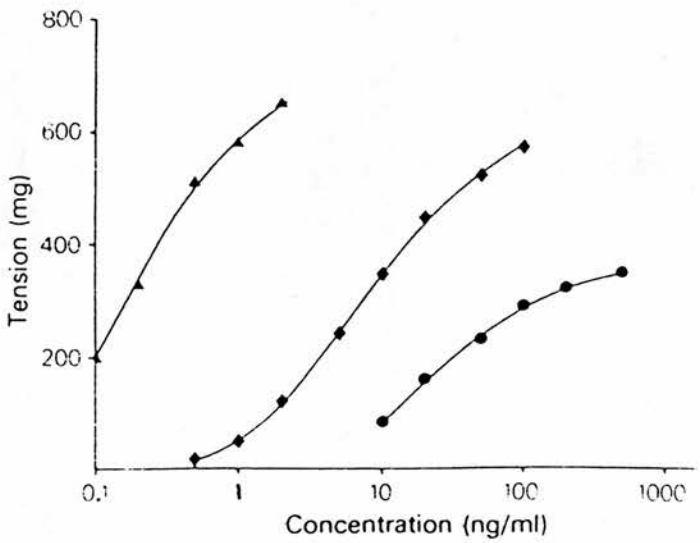


Figure E.5 Bullock iris sphincter muscle log concentration-response curves for 11,9-epoxymethano PGH_2 (◆), EP 011 (▲), and 9, 11-ethano PGH_2 (●).

Table E.2 Relative potencies of thromboxane analogues on the bullock isolated iris sphincter preparation

Substance	Equipotent molar ratio (Standard agonist 11,9 epoxymethano PGH_2 = 1.0)			
	Individual value			mean ± s.e.
(±) EP 011	0.010	0.013	0.020	0.033 ± 0.0074
	0.020	0.030	0.044	n = 9
	0.057	0.060	0.070	
(±) 15 oxo EP 011	0.027	0.030	0.039	0.034 ± 0.0032
	0.040			n = 4
9,11-azo PGH_2	0.39	0.62	0.96	0.74 ± 0.15
	1.00			n = 4
CTA_2	0.50	0.54	1.28	0.77 ± 0.25 n=3
(±) 9,11 ethano PGH_2	50%	66%	70%	p.a. 66 ± 6%
	79%			
	0.21	0.31	0.41	0.40 ± 0.10 (μM)
	0.68(μM)			
PTA_2	44%	50%	-	
	0.080	0.29	0.32(μM)	
(±) ICI 79939 $PGF_{2\alpha}$	0.81			

Individual values are the result of a comparison with 11,9-epoxymethano PGH_2 on a single preparation. With partial agonists on TxA_2-sensitive sites, values of the relative maximum response (11,9-epoxymethano PGH_2=100%) together with the concentration of the partial agonist required to produce a response 50% of its own maximum are given.

giving half of its own maximum response and the molar concentration of the test compound producing an equivalent response. For a partial agonist the maximum response relative to that of 11,9-epoxymethano PGH2 is given together with the concentration which produced a response 50% of the partial agonist maximum. EP Ø11 and 15-oxo EP Ø11 are the most potent contractile agents. The threshold concentration for EP Ø11 is about 40 pg/ml.

There is some difficulty in establishing the absolute maximum response using a cumulative dose regime when the on-set of an agonist is slow. We particularly wished to know whether CTA2 is a full agonist or not, so a single high concentration of CTA2 (1 µg/ml) was added to the organ bath. which showed that CTA2 reached the same maximum as 11,9-epoxymethano PGH2 did. The 9,11-ethano PGH2 analogue and PTA2 were less active than 11,9-epoxymethano PGH2 and gave a lower maximum response than EP Ø11 or 11.9-epoxymethano PGH2 maxima (see Figure E.5). They opposed the contractile action of 11.9-epoxymethano PGH2, but their contractile actions were additive with that of PGE2.

Effects of the Thromboxane Antagonists EP Ø45 and EP 116

In a series of experiments the effects of EP Ø45 and EP 116 on the contractile action of several of the analogues was determined.

It was considered essential to run control tissues simultaneously to ensure that there was little change in agonist sensitivity with time on the bullock iris sphincter

preparation. On control preparations three cumulative concentration-response relationships for PGE2 or 11.9-epoxymethano PGH2 were established at approximately hourly intervals. In the antagonist-treated preparations EP 045 at 1.3 μM or EP 116 at 0.034 μM was added 5-10 min before the start of the second agonist sequence and further increased to 2.6 μM for EP 045 or 0.068 μM for EP 116 5-10 min before the start of the third sequence. These antagonists caused a pronounced parallel shift to the right of the 11.9-epoxymethano PGH2 log concentration-response curve (Figure E.6). Dose-ratios are shown in Table E.3.

From the Schild equation are obtained affinity constants of 6.9 (s.e.mean $\pm$ 0.9) x 10^6 and 7.5 ($\pm$ 0.7) x 10^6 M^{-1}, respectively for the lower and higher concentrations of EP 045. and affinity constants of 7.60 ($\pm$ 1.05) x 10^8 and 8.05 ($\pm$ 0.94) x 10^8 M^{-1}, respectively for the lower and higher concentrations of EP 116. EP 045 or EP 116 caused much smaller shifts of the PGE2 concentration-response curves (see Table E.3). More determinations would be required to determine whether this small degree of antagonism is significant. The agonist actions of ZK 36374 and PGI2 were not blocked by EP 045, and EP 045 and EP 116 had little effect on PGF2a and ICI 79939 PGF2a.

With 16.16-dimethyl PGE2 the situation is more complex. The log concentration-response curve for 16,16-dimethyl PGE2 over the concentration range of 0.1-20 ng/ml was parallel to that of PGE2 (see Figure E.2). and remained constant during the experiment. Under these circumstances EP 045 (1.3 μM)

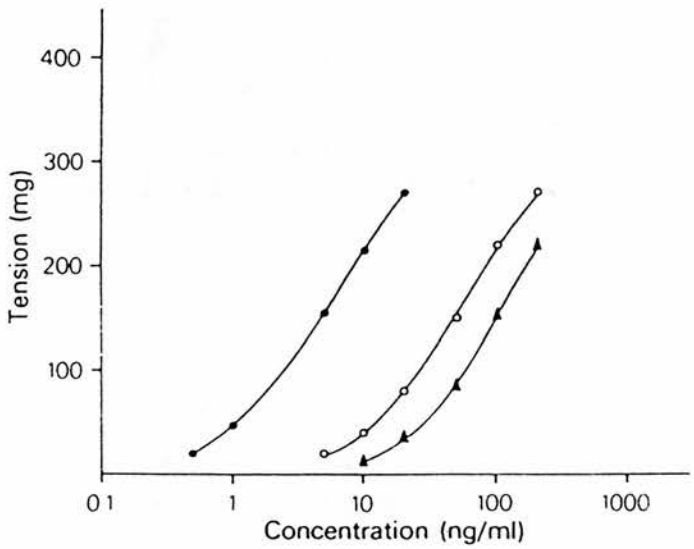


Figure E.6 Bullock iris sphincter muscle: log concentration-response curves for 11,9-epoxymethano PGH_2 alone (●) and in the presence of 0.5 (○) and 1.0 (▲) μg/ml EP 045.

Table E.3 Effects of EP 045 or EP 116 on contractile response to different agonists on the bullock iris sphincter

Treatment	Agonist	No. of tests	Dose-ratios for agonist cumulative sequences (first sequence = 1.00) second	third
Control	PGE_2	4	0.86 ± 0.16	0.96 ± 0.18
	16,16-dimethyl PGE_2		1.39 1.50	
	PGI_2		1.00 1.10 1.56	
	11,9-epoxymethano PGH_2	4	1.06 ± 0.07	1.27 ± 0.13
EP 045	PGE_2	3	1.16 ± 0.03	1.63 ± 0.13
	16,16-dimethyl PGE_2	5	1.72 ± 0.15	3.2
	PGI_2		1.47	
	11,9-epoxymethano PGH_2	4	10.0 ± 1.20	20.5 ± 1.80
	ZK 36374		0.83 1.10	1.00
	$PGF_{2\alpha}$		2.39	
EP 116	PGE_2		0.94* 1.21*	
	11,9-epoxymethano PGH_2	4	27.0 ± 3.50	56.0 ± 6.30
	(±)ICI 79939 $PGF_{2\alpha}$		2.60	

With the antagonist - treated preparations the second agonist sequence was established in the presence of 1.3×10^{-6} M EP 045 or 3.4×10^{-8} M EP 116 and the third sequence in the presence of 2.6×10^{-6} M EP 045 or 6.8×10^{-8} M EP 116.

* indicates that a dose-ratio was obtained in the presence of 1.1×10^{-6} M EP 116.

produced only a small shift of the 16,16-dimethyl PGE2 curve (see Table E.3). At higher concentrations of 16,16-dimethyl PGE2 (Ø.1-2.Ø μg/ml) further small contractions could be elicited which eventually approached the 11.9-epoxymethano PGH2 maximum (Figure E.7). However, in this situation some tachyphylaxis to the contractile action of 16,16-dimethyl PGE2 occurred, such that subsequent concentration-response curves were shifted to the right, with slight lowering of the maximum response (Figure E.7b). This made any effect of EP Ø45 on the high concentration component of 16,16-dimethyl PGE2 action difficult to determine (Figure E.7a).

As to EP Ø11 and 15-oxo EP Ø11, when a complete cumulative log concentration-response relationship has been obtained on a preparation, the response returned slowly to baseline over a period of at least 3 hours during repeated wash-out of the organ bath. Re-testing of the standard agonist showed that there was some loss of sensitivity. Therefore, with these compounds, estimates of their relative contractile activities in the presence and absence of EP 116 were made using the log concentration-response curves for 11,9-epoxymethano PGH2 and EP Ø11 or 15-oxo EP Ø11 on two preparations: one was treated with 11.9-epoxymethano PGH2 and then with either EP Ø11 or 15-oxo EP Ø11 to establish their cumulative dose-response relationships; on the other preparation the procedure was to set up cumulative concentration-response relationships for both PGE2 and 11,9-epoxymethano PGH2, wash out the agonists, expose the tissue to a fixed concentration of EP 116 for 1Ø-2Ø min and

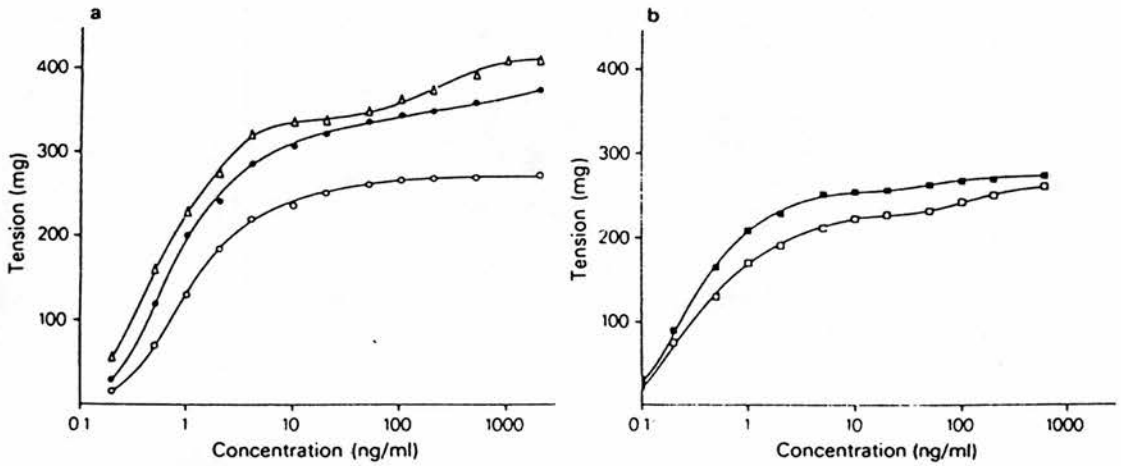


Figure E.7 Bullock iris sphincter muscle: (a) Preparation A—log concentration-response curve for 16,16-dimethyl PGE_2 (Δ) showing distinctly the two components of the curve. Preparation B—concentration-response relationships for 16,16-dimethyl PGE_2 in the absence (●) and the presence (○) of EP 045 (0.5 μg/ml). (b) Preparation C—control tissue on which two consecutive cumulative concentration-response relationships for 16,16-dimethyl PGE_2 were established (■, □).

then set up a further dose-response relationship for either EP Ø11 or 15-oxo EP Ø11. 11,9-epoxymethano PGH2 was taken as a standard agonist, dose ratios for EP Ø11 or 15-oxo EP Ø11 with and without the antagonist were worked out by the following formula:

$$\text{Dose Ratio} = \frac{\text{EC5Ø EP Agonist with EP 116}}{\text{EC5Ø 11,9-EM PGH2}} \times \frac{\text{EC5Ø 11,9-EM PGH2}}{\text{EC5Ø EP Agonist}}$$

Here EP Agonist indicates EP Ø11 or 15-oxo EP Ø11 and 11,9-EM PGH2 indicates 11,9-epoxymethano PGH2.

In the absence of EP 116, EP Ø11 and 15-oxo EP Ø11 were very active and of equal potency, and both of them had a consistent relationship with 11,9-epoxymethano PGH2 (see Table E.2). The equipotent molar ratios between 11,9-epoxymethano PGH2 alone and the combination of EP Ø11 or 15-oxo EP Ø11 with 1.1 μM EP 116 varied to a large extent. This was 1.2-15 for EP Ø11 and 5.4-33 for 15-oxo EP Ø11. Hence the calculated dose ratios of EP Ø11 or 15-oxo EP Ø11 with and without EP 116 covered a large range: 36-46Ø for EP Ø11 and 16Ø-97Ø for 15-oxo EP Ø11. However, concentration-response curves for EP Ø11 or 15-oxo EP Ø11 in the presence of 1.1 μM EP 116 had a relatively consistent relationship with that of PGE2. Using PGE2 as a standard agonist, the dose ratios were 14, 19, 25 for EP Ø11 with EP 116 present and 63, 88, 1ØØ for 15-oxo EP Ø11 with EP 116 present. EP 116 at the concentration of 1.1 μM caused little shift of the PGE2 concentration-response

curve (see Table E.3).

Effects of ICI 79939 PGF2a, ICI 799339 PGE2 and M/B 28767

ICI 79939 PGF2a, a PGF2a analogue, is very active on the bullock iris sphincter preparation. The equipotent molar ratio for ICI 79939 PGF2a is 0.77 (PGE2=1.0) and 0.81 (11,9-epoxymethano PGH2=1.0). EP 116 (1.1 μM) caused a small shift to the right of the ICI 79939 PGF2a log concentration-response curve (dose ratio=2.6). And adding EP 045 (2.6 μM) against the background of a contractile response being produced with M/B 28767 brought about a marked fall of the response. Thus the experiment for measuring EPMR of ICI 79939 PGF2a, ICI 79939 PGE2 and M/B 28767 were carried out in the presence of a fixed concentration of either EP 045 or EP 116 in order to block TxA2 activity of these compounds. Equipotent molar ratios are shown in Table E.4. PGE2 in the presence of either EP 045 or EP 116 was used as the standard. Log concentration-effect curves for ICI 79939 PGF2a and ICI 79939 PGE2 were parallel to that of PGE2 (Figure E.8) while the log concentration-effect curve of M/B 28767 took on a flat shape: its threshold concentration was less than 1 ng/ml; its 'maximum response' concentration was more than 1 μg/ml and its curve reached a higher maximum than PGE2 (Figure E.9).

Effects of Indomethacin

In the absence of indomethacin, the tone of the bullock iris sphincter preparation remained stable for at least 1 h. After challenge with prostanoids, in particular with PGE2

Table E.4 Activities of ICI 79939 $PGF_{2\alpha}$, ICI 79939 PGE_2 and M/B 28767 in the presence of EP 045 or EP 116 on the bullock iris sphincter preparations.

Compound	TxA_2 receptor antagonist (μM)	Equipotent molar ratio (Standard agonist PGE_2=1.0 in the presence of EP 045 or EP 116)
(±) ICI 79939 $PGF_{2\alpha}$	EP 116 (1.1)	2.0
	EP 045 (1.3)	1.2
	EP 045 (2.6)	1.5 1.6 2.2
(±) ICI 79939 PGE_2	EP 045 (2.6)	0.17 0.17
(±) M/B 28767	EP 045 (2.6)	10 12 14
	EP 045 (5.2)	39

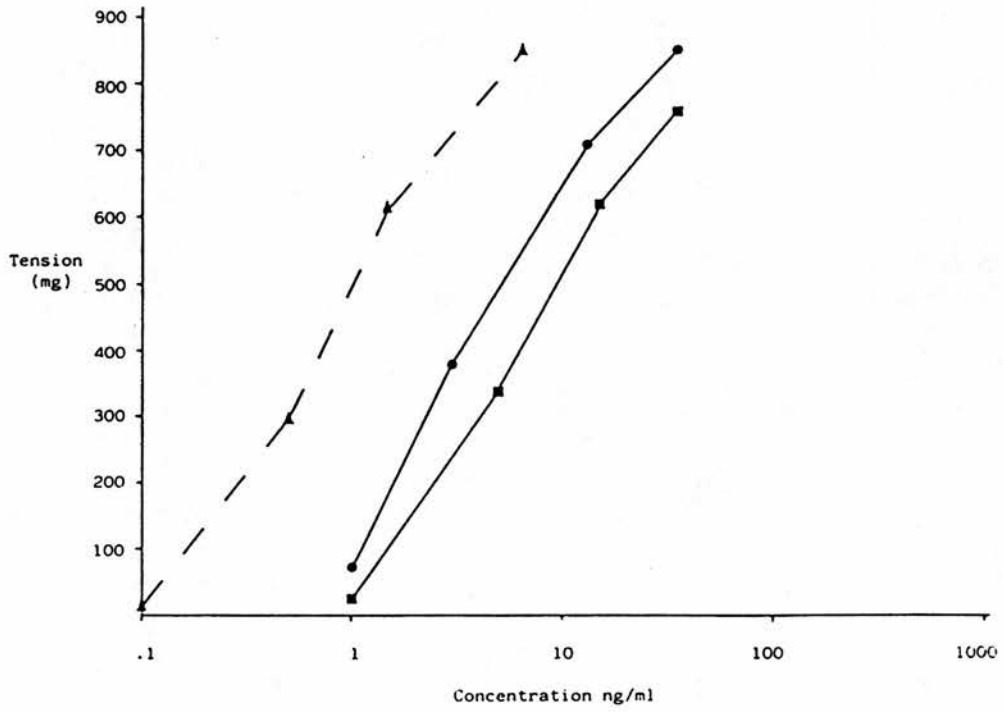


Fig. E.8 Bullock iris sphincter muscle: Log concentration-response curve for PGE_2 (●). ICI 79939 PGE_2 (▲) and ICI 79939 $PGF_{2\alpha}$ (■) in the presence EP 045 (1 µg/ml).

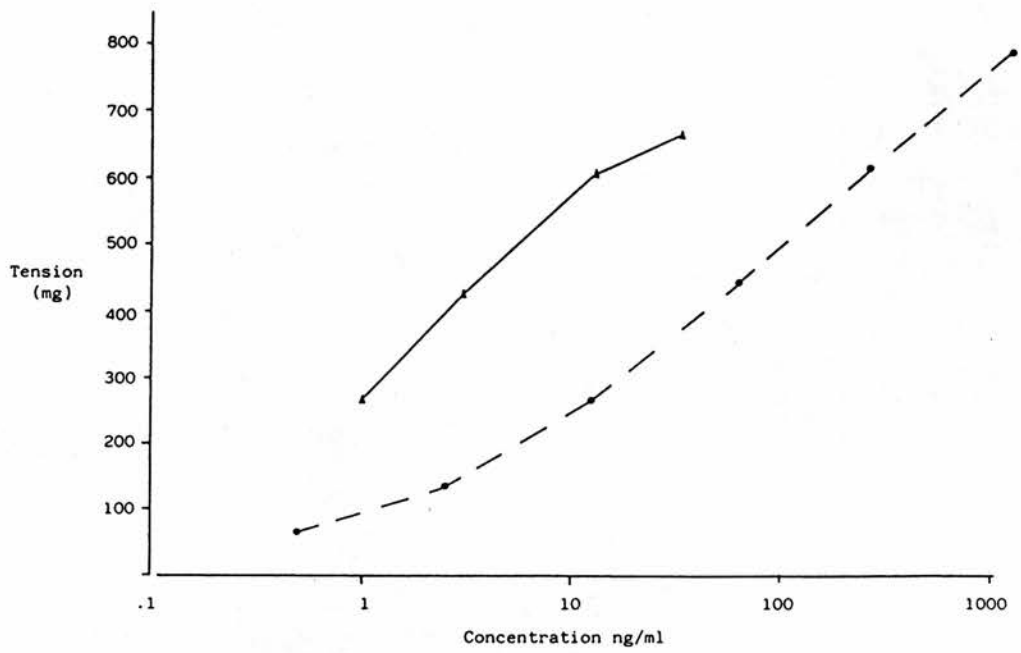


Fig. E.9 Bullock iris sphincter muscle: Log concentration-response curve for PGE_2 (▲) and M/B 28767 (●) in the presence of EP 045 (1 µg/ml) and indomethacin (10^{-6} M).

and using Krebs solution as a bath solution, the basal tone would increase gradually. Atropine or phentolamine failed to depress the rise in tone. Indomethacin (1 μM) lowered the tone and this remained constant throughout the remainder of the experiment. In order to find out whether indomethacin affects responses to PGs, the contractions produced by some prostanoids were measured both in the absence and presence of indomethacin. The results are shown in Table E.5. The first sequence was in the absence of indomethacin. Following wash-out the tissue was exposed to a fixed concentration of indomethacin for 20 min, then a further dose-response relationship for agonists was established. Following the same procedure, the third and fourth sequences were established.

Effects of EP 045 and EP 116 on the spontaneously increased tone were also investigated. EP 045 (1.3-5.2 μM) inhibited the tone dose-dependently; on the other hand EP 116 2.2 μM had little effect on the tone.

Effects of TxA2 and PGH2

Effects of synthetic PGH2 and TxA2 generated enzymatically from synthetic PGH2 were studied using the cascade technique. Pairs of the bullock iris sphincter preparation arranged in series, were challenged with TxA2 and PGH2. For the generation of TxA2 a given amount of PGH2 in Krebs solution was mixed with a suspension of horse platelet microsomes (HPM), a rich source of the enzyme thromboxane synthetase (Moncada, Needleman, Bunting & Vane, 1976), and incubated for 30 s at 0°C. GC/MS analysis, using authentic

Table E.5 Effect of indomethacin on contractile responses to different agonists on the bullock iris sphincter preparation

Agonist	Sequence	Indomethacin (M)	Dose-ratios for cumulative sequences (first sequence=1.0 in the absence of indomethacin)
11,9-epoxymethano PGH_2	second	$2X10^{-6}$	0.76 1.3
	third	$4X10^{-6}$	0.70 1.0
	fourth	10^{-5}	0.90 1.0
PGE_2	second	10^{-6}	1.4
	third	$5X10^{-6}$	2.4
16,16-dimethyl PGE_2	second	10^{-6}	1.4
	third	10^{-5}	4.6

TxB2 as an external standard. showed that during the 30 s incubation period about 60% of the PGH2 had been converted to TxA2 (Armstrong, Jones & Wilson, 1983). The assay tissues were treated with a mixture of blocking agents to reduce or abolish the actions of acetylcholine, histamine, 5-hydroxytryptamine (5-HT) and catecholamines. Indomethacin (3 μM) was also added to prevent thromboxane and prostaglandin production in the assay tissues themselves. Before PGH2 and TxA2 were superfused into the cascade system, dose-response relationships for both 11,9-epoxymethano PGH2 and PGE2 were established. Submaximum doses of 11.9-epoxymethano PGH2 (150 ng) and PGE2 (100 ng), superfused for 30 s (flow rate=10 ml/min), were chosen.

In order to antagonize PGE2 activity, ZK 36374, a partial agonist on PGE2 receptor sites, was superfused continuously at a final concentration of 250-500 ng/ml. ZK 36374 gave a submaximum contraction and blocked the effect of PGE2 but not 11.9-epoxymethano PGH2.

Sufficient generated TxA2 was superfused onto the preparations to give a response which matched that produced by the above dose of 11.9-epoxymethano PGH2.

The further identification of the generated component as TxA2 was based on the known short half-life of TxA2 in aqueous solution at pH 7.6 and room temperature (22°C). Allowing the generated TxA2 to stand at room temperature for 30-45 s caused a marked reduction in the contractile response (Figure E.10-11).

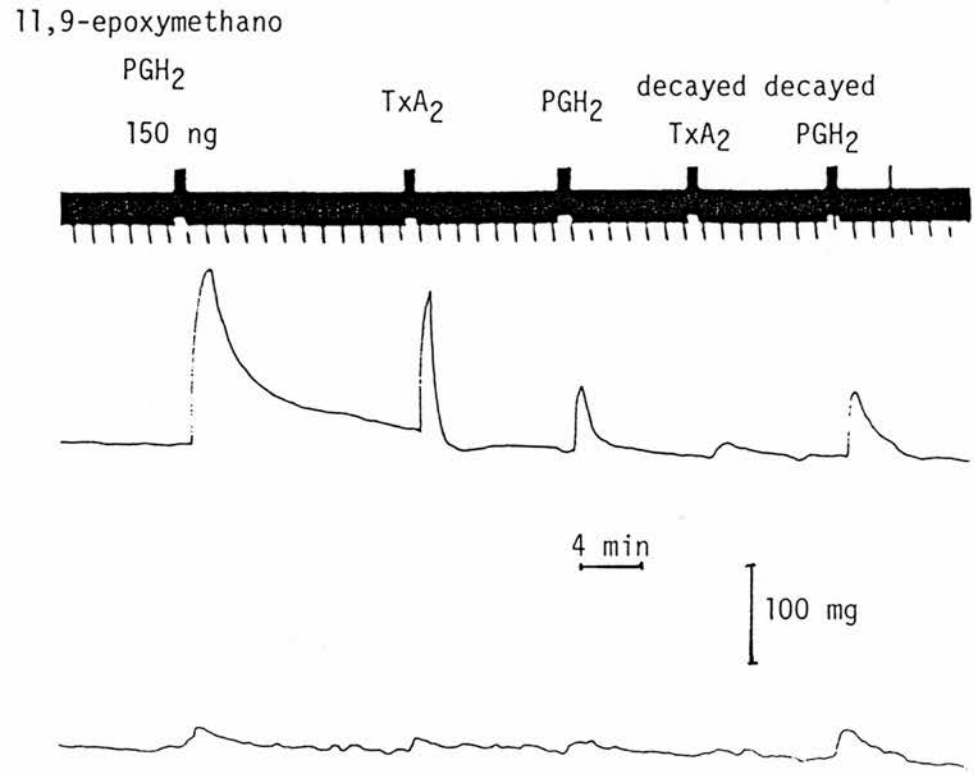


Figure E.10-11 Bullock iris sphincter preparations:
Effects of PGH_2 and generated TxA_2. The records show tension changes in two bullock iris sphincter preparations superfused in series. ZK 36374 (500 ng/ml) was applied to both preparations and EP 045 (1 µg/ml) to the second preparation in the cascade (lower record) (flow rate = 10 ml/min). A given concentration of PGH_2 was chosen, and mixed with HPM to generate TxA_2. Generated TxA_2 and PGH_2 were left at room temperature for 45 s and 60 s, respectively, to decay.

HPM, see P 34.

Treatment of the second tissue in the cascade with EP 045 (final concentration (2.6 μM) inhibited responses to the generated TxA2, PGH2 and the 11,9-epoxymethano PGH2 analogue. Typical experiments are shown in Figure E.10-11. The generated TxA2 produced a stronger effect than the original amount of PGH2 (see Figure E.10-11).

Dog and Cat Iris Sphincter Muscle

A limited number of dog and cat iris sphincter preparations were available for testing of the prostanoids. PGF2a was a potent contractile agent on both preparations. Responses to PGF2a reached a plateau after 2-10 min and then gradually declined. ICI 81008, ICI 79939 PGF2a, ICI 79939 PGE2. ICI 80205 and EP 011 had relatively slow on-sets of action.

PGF2a produced a 50% maximum response at concentrations of 0.3-1.5 ng/ml on the dog iris sphincter muscle and 2.9-5.3 ng/ml on the cat iris sphincter muscle. Table E.6 summarizes the relative activities of the prostanoids tested. All the compounds in the table showed contractile activity. There was no evidence for a prostaglandin-induced inhibitory effect on either of these preparations. ICI 81008 gave the same maximum as PGF2a did both in the dog and in the cat iris sphincter preparation, and showed the highest activity of all the compounds tested. ICI 79939 PGF2a was about twice as potent as PGF2a on the dog preparation.

ZK 36374 and PGI2 were very weak contractile agents. The actions of ZK 36374 and PGF2a were additive as shown in

Table E.6 Activities of prostanoids on the dog and cat isolated iris sphincter preparations

Compound	Equipotent molar ratio (PGF_2 =1.0)	
	Dog	Cat
(±)ICI 81008	0.10 0.19 0.49	0.19 0.19 0.19 0.19 (0.19±0.00,n=4)*
(±)ICI 79939 $PGF_{2\alpha}$	0.24 0.31 0.86	
16,16-dimethyl $PGF_{2\alpha}$	1.2 1.5	
13,14-didehydro $PGF_{2\alpha}$	2.7 4.5	
PGD_2		20 53
PGI_2	(3200) (3400)	
ZK 36374	(120) (>100) (1200) 900	(490)
PGE_2	60 137 230	40 40 45 45 46 54 (45±2.1, n=6)*
16,16-dimethyl PGE_2	530 530 (>1000)	81 105 122 124 144 (115±11, n=5)*
(±)ICI 80205	3.1 4.2 5.9 (4.4±0.81, n=3)*	1.7 2.2 2.9 3.2 (2.5±0.34, n=4)*
(±)ICI 79939 PGE_2		2.4 3.1 3.1 (2.9±0.22, n=3)*
(±)11-deoxy PGE_1	(>2000)	
11,9-epoxymethano PGH_2	(120) (230) (300)	(100)
(±)EP 011	11	

Individual values are the result of a comparison with $PGF_{2\alpha}$ on a single preparation. Values in parentheses are derived from experiments in which a complete concentration-response curve for the test compound was not established, equipotent molar ratios relate to responses 20% of the $PGF_{2\alpha}$maximum.

Figures in the brackets marked with * indicate mean±s.e., n= number of test.

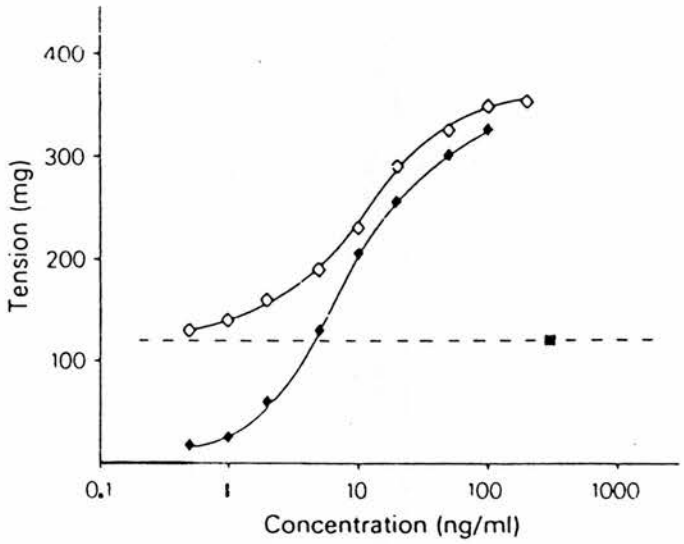


Figure E.12 Dog iris sphincter preparation: log concentration-response curve for $PGF_{2\alpha}$ acting alone (◆) and the corresponding curve (◇) in the presence of 0.3 μg/ml ZK 36374 (■).

Figure E.12.

Some PGE2 analogues were also tested. Of particular interest is the low activity of 16,16-dimethyl PGE2 on both preparations, and the high potency of ICI 80205 and ICI 79939 PGE2.

On five preparations EP 045 at 500 ng/ml (1.3 μM), on four preparations EP 092 at 1.0 ug/ml (2.4 μM), and on two preparations EP 116 at 500 ng/ml (1.1 μM) produced only small shifts of the PGF2a log concentration-response curve (Table E.7).

11,9-epoxymethano PGH2 had weak activity. Its full concentration-response curve was not established. EP 045 did not block the effect of 11.9-epoxymethano PGH2 on the dog iris sphincter preparation.

Other TxA2 Analogues

Six compounds with TxA2-like activity were tested. They were 9,11-azo PGH2, 9,11-ethano PGH2, 15-oxo EP 011, 15 α EP 130, 15 β EP 130, 15-oxo EP 130 and PTA2. All the compounds except PTA2 showed a contractile effect. The effect of EP 116 was tested on 9,11-ethano PGH2 and 15-oxo EP 011. EP 116 at concentration of 500 ng/ml did not block their contractile effects. 9,11-Azo PGH2, 9,11-ethano PGH2, 15-oxo EP 011, 15-oxo EP 130, 15 α EP 130 and 15 β EP 130 were agents which displayed slow on-sets and gave a maximum contractile response lower than that produced by PGF2a. Their values of the relative maximum response (PGF2a=100%) together with their EC50 values (concentrations required to

Table E.7 Effects of EP 045 and EP 116 on contractile responses to $PGF_{2\alpha}$ and 11,9-epoxymethano PGH_2 on the dog iris sphincter preparation

Treatment	Agonist	Number of test	Dose-ratios for agonist cumulative sequences (first sequence=1.0) Second
Control	$PGF_{2\alpha}$	5	1.0 1.0 1.2 1.4 1.8 (1.3±0.15)
EP 045	$PGF_{2\alpha}$	5	1.4 1.5 1.8 1.8 2.8 (1.9±0.25)
EP 045	11,9-epoxymethano PGH_2	2	1.0 1.3
EP 116	$PGF_{2\alpha}$	2	0.86 1.6
EP 092	$PGF_{2\alpha}$	4	1.4 1.4 1.6 1.7 (1.5±0.075)

The first sequence was in the absence of TxA_2 receptor antagonists. With TxA_2 receptor antagonist-treated preparations the second agonist sequence was established in the presence of $1.3X10^{-6}$ M EP 045, $1.1X10^{-6}$ M EP 116 or $2.4X10^{-6}$ M EP 092.

produce a response 50% of its own maximum) are shown in Table E.8. In terms of EC50, 15-oxo EP Ø11 is the most potent among the compounds above, and PTA2 in concentrations of up to 2ØØ ng/ml showed no contractile activity. 15 α EP 13Ø was more active than 15 β EP 13Ø. 9,11-Azo PGH2 produced a much lower maximum response relative to that of PGF2a.

Activities of those compounds against PGF2a were also examined, by adding cumulative doses of PGF2a to the organ bath in the presence of a fixed concentration of one of the compounds. PTA2 at 1 µg/ml did not affect the response to PGF2a. 9,11-Azo PGH2 and 9,11-ethano PGH2 showed additive interactions with PGF2a. In contrast 15-oxo EP Ø11, 15 α EP 13Ø, 15 β EP 13Ø and 15-oxo EP 13Ø opposed the effect of PGF2a (Figure E.13). In most cases, PGF2a in the presence of one of the above four compounds failed to reach the maximum response obtained with PGF2a alone.

The responses to 15-oxo EP Ø11 was intensively studied since it was the most active compound. With low concentrations of PGF2a (<2 ng/ml) and 15-oxo EP Ø11 (<5Ø ng/ml) responses remained stable for 1Ø-2Ø min after the responses reached their plateau. However, responses to higher concentrations of PGF2a or 15-oxo EP Ø11 faded (Figure E.14). Falling rates of responses to PGF2a and 15-oxo EP Ø11 are listed in Table E.9. The fall occurred 4-6 min after the response to 15-oxo EP Ø11 (1ØØ ng/ml) had peaked and Ø.5-1 min after the response to PGF2a (>5 ng/ml) reached its peak. Higher concentrations caused a more rapid fall of the response.

Table E.8 Activities of TxA2 analogues on isolated dog iris sphincter preparations.

Compound	Lower and upper values of the relative maximum response ($PGF_{2\alpha}$=100%)	EC_{50} (ng/ml)
(±)15-oxo EP 011	20-70% (20 30 45 50 50 50 50 50 50 50 50 60 60 60 64 70 70%)	3.4 5.0 5.0 6.0 10 10 11
15α EP 130	10-40% (10 32 40%)	36 50
15β EP 130	25-50% (25 50%)	500 1500
(±)15-oxo EP 130	40-60% (40 50 60%)	60 100
(±)9,11-ethano PGH_2	40-88% (40 60 60 70 75 88%)	100 100 150 180 180
9,11-azo PGH_2	10-20% (10 15 20%)	100 100 140

Values in parentheses indicate single values of the relative maximum response.

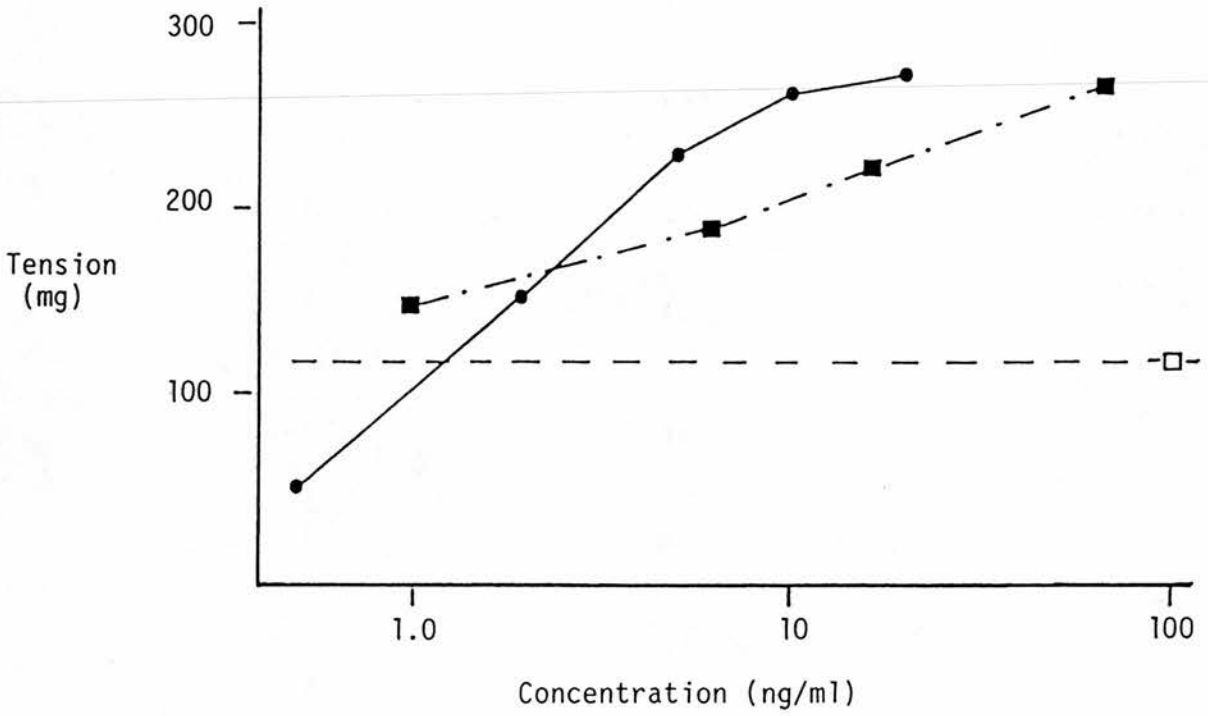


Figure E.13 Dog iris sphincter muscle: interaction of 15-oxo EP 011 with $PGF_{2\alpha}$. A cumulative concentration-response relationship was first established to the $PGF_{2\alpha}$ (solid circle), followed by a cumulative relationship to the $PGF_{2\alpha}$ (solid square) in the presence of a fixed concentration of 100 ng/ml 15-oxo EP 011 (open square).

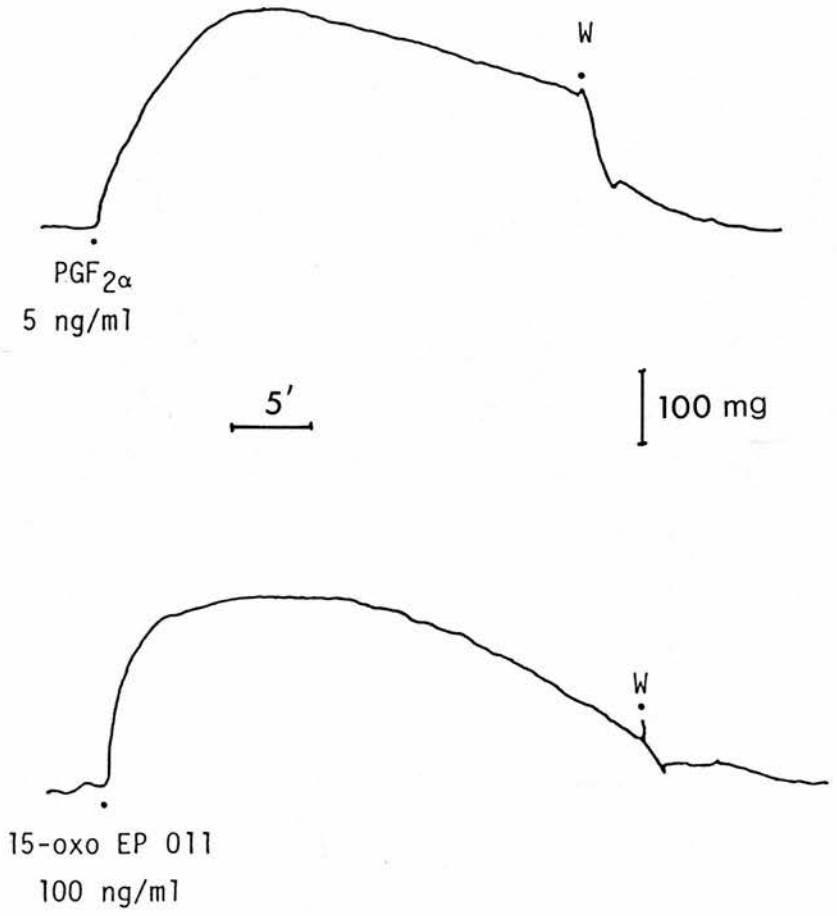


Figure E.14 Dog iris sphincter preparations: spontaneous fading of responses to $PGF_{2\alpha}$ (upper trace) and 15-oxo EP 011 (lower trace). W = wash.

Table E.9 Falling rates of responses to $PGF_{2\alpha}$ and 15-oxo EP 011 on the dog iris sphincter preparation.

Compound	Dose (ng/ml)	Falling rate (mg/min)
$PGF_{2\alpha}$	5	5.0 6.0
	10	10 14
(±)15-oxo EP 011	100	8.1 9.0 9.0 10 11 20

The falling rate was measured by finding the point where the response began to fall, counting the fall (mg) within a certain time and dividing the fall by the time (min).

Figure E.15a and 15b shows that prolonged treatment of the preparation with PGF2a or 15-oxo EP Ø11 resulted in loss of sensitivity to PGF2a. The sensitivity after treatment with PGF2a 2Ø ng/ml for 2Ø min was restored within 2 hours, but the recovery of sensitivity in 15-oxo EP Ø11-treated preparations took a much longer time.

The interaction of 15-oxo EP Ø11 with carbachol was also studied and the contractile action of carbachol was obviously additive with that of 15-oxo EP Ø11.

Horse Iris Sphincter Muscle

PGF2a Analogues

PGF2a showed contractile activity on the horse iris sphincter preparations similar to that found on the dog and cat iris sphincter muscle. Responses to PGF2a peaked at 2-4 min and then declined slowly. The falling rate was Ø.5-2.Ø mg/min. and increasing the concentration of PGF2a did not accelerate the fall of response.

PGF2a elicited a 5Ø% maximum response at a mean bath concentration of 7.5 $\pm$ s.e.Ø.84 ng/ml (n=38). The threshold concentration which gave tension change of about 2Ø mg was Ø.87 $\pm$ s.e.Ø.15 ng/ml (n=38). The relative activities of several other prostanoid analogues are shown in Table E.1Ø. ICI 81ØØ8 and ICI 79939 PGF2a exhibited relatively slow on-sets of action and peaked at 6-1Ø min. EP 116 (1.1 μM) caused little shift to the right of the PGF2a log

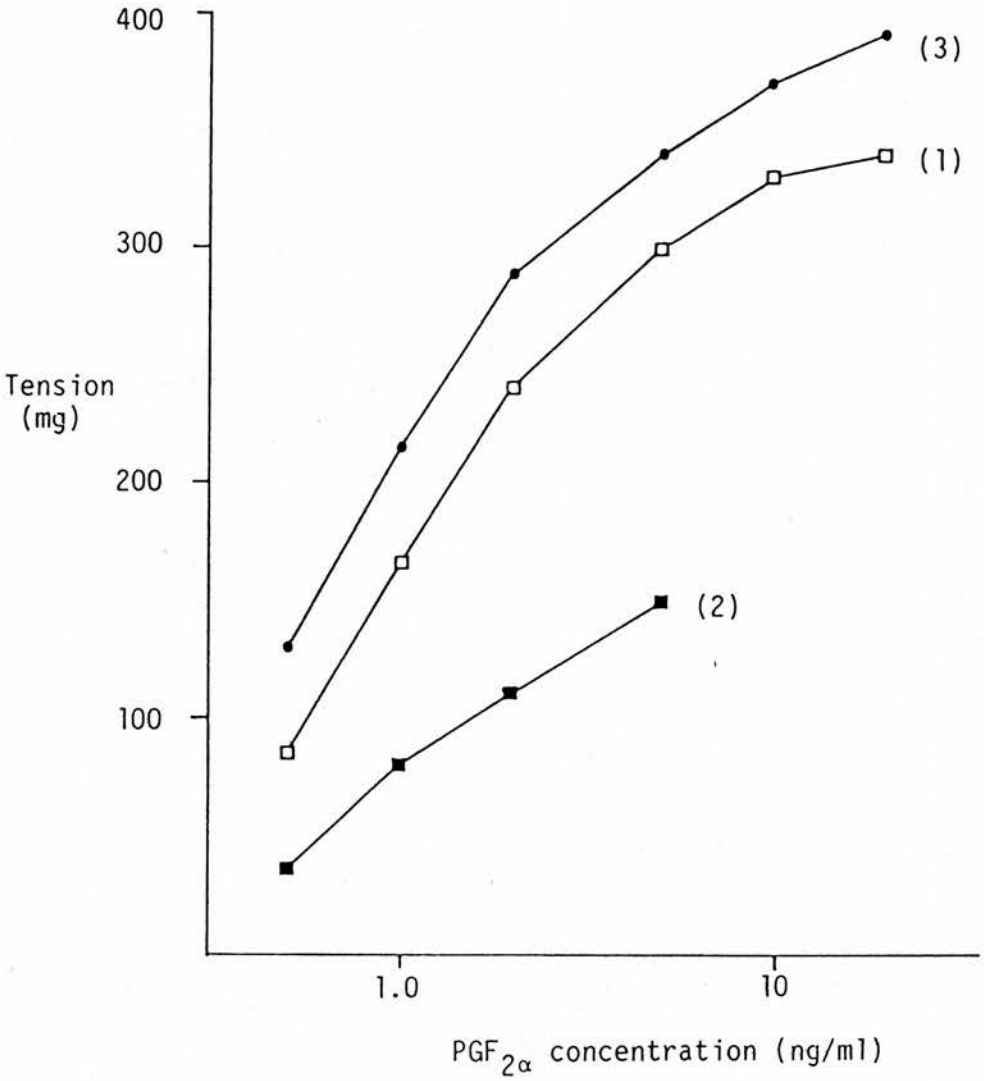


Figure E.15a Dog iris sphincter muscle: desensitization to $PGF_{2\alpha}$. Three consecutive sequences of cumulative concentration-response relationship for $PGF_{2\alpha}$ were established on a single preparation. The second (2) and third (3) sequences were commenced 10 min and 2 h, respectively, after exposure to 20 ng/ml PGF2α for 20 min, and the first (1) was prior to the treatment. The preparation picked up sensitivity to $PGF_{2\alpha}$ in the third sequence.

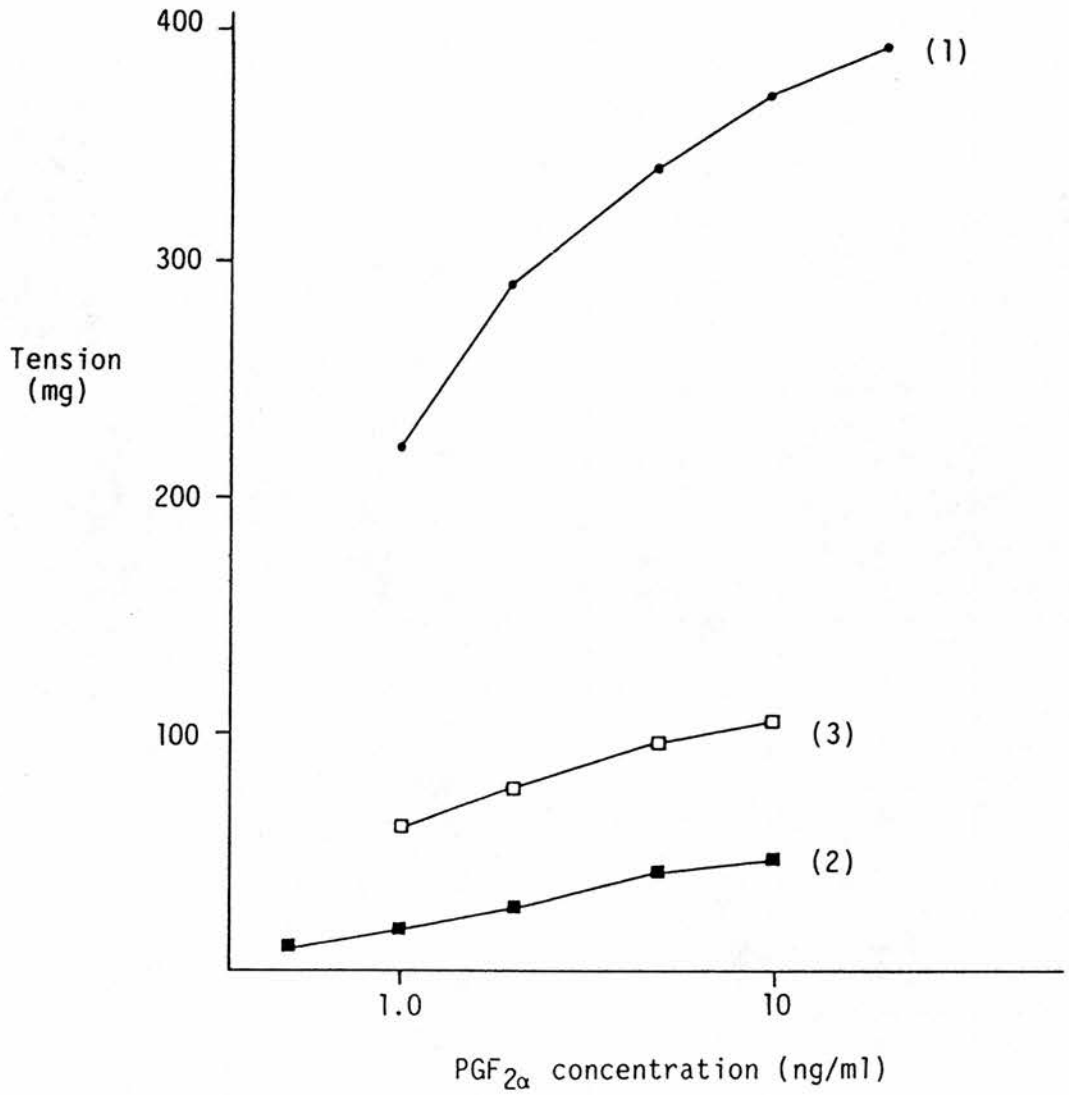


Figure E.15b Dog iris sphincter muscle: desensitization by 15-oxo EP 011. Three consecutive sequences of cumulative concentration-response relationship for $PGF_{2\alpha}$ were established on a single preparation. The first sequence (1) was prior to 15-oxo EP 011 treatment, and the second (2) and third (3) commenced 1.5 and 2 h, respectively, after exposure to 15-oxo EP 011 100ng/ml for 20 min.

Table E.10 Activities of prostanoid analogues on the horse iris sphincter preparation.

Compound	Equipotent molar ratio individual value	mean±s.e.	n
$PGF_{2\alpha}$ related drugs	($PGF_{2\alpha}$=1.0)		
(±)ICI 79939 $PGF_{2\alpha}$	0.14 0.18 0.19 0.25	0.19±0.023	4
(±)ICI 81008	0.21 0.23 0.33 0.36	0.28±0.037	4
(±)ICI 80205	2.38 3.25 3.25	3.0±0.29	3
PGD_2	55 65 66	62±3.5	3
11,9-epoxymethano PGH_2	(400) (660)		
9,11-azo PGH_2	(2800)		
PGE_2 related drugs			
Contractile effect	(PGE_2=1.0)		
16,16-dimethyl PGE_2	0.25 0.26 0.27 0.29 0.55 0.56	0.36±0.061	6
Relaxant effect	(PGE_2=1.0)		
PGE_2 (second sequence)	1.3		
PGE_1	0.28 0.71		
PGI_2	70 80		
ZK 36374	98		

Individual values are the result of a comparison with $PGF_{2\alpha}$ or PGE_2 on a single preparation. Values in parentheses are derived from experiments in which a complete concentration-response curve for the test compound was not established; equipotent molar ratios related to responses 20% of the $PGF_{2\alpha}$ maximum.

dose-response curve (dose ratio=1.6). ICI 81008 was 3-5 times more potent than PGF2a, as seen in the dog and cat iris sphincter preparations, but gave a slightly lower maximum response than that produced by PGF2a. In contrast, ICI 79939 PGF2a, the most active compound among the drugs tested, produced a maximum contractile response higher than that obtained with PGF2a (mean=139%, range 120-180%, n=4) (Figure E.16). There was no evidence that PGF2a produced a relaxant effect.

PGE2 and PGI2 Analogues

The preparation possessed intrinsic tone (1.0 g), which indomethacin 1 μM could not completely abolish. In the presence of indomethacin 1 μM the preparation provided a tone equivalent to 100-500 mg.

The PGE2 and PGI2 analogues showed different properties from PGF2a on the horse iris sphincter preparation. On 18 out of 22 preparations, PGE2 produced a relaxant response at concentrations of 0.5-10 ng/ml; at higher concentration of PGE2, a reversal of the relaxant action was seen (Figure E.17). The extent of the PGE2-induced relaxant effect was not obviously related to the amount of intrinsic tone present. Since PGE2 produced dual effects the equipotent molar ratio relative to PGF2a could not be measured. From an experiment with PGF2a and PGE2 on a single preparation which did not markedly relax to PGE2 it was found that the contractile activity of PGE2 was 10-20 times less than that of PGF2a(Figure E.18a). Of interest is the activity of 16,16-dimethyl PGE2. It produced a maximum contractile

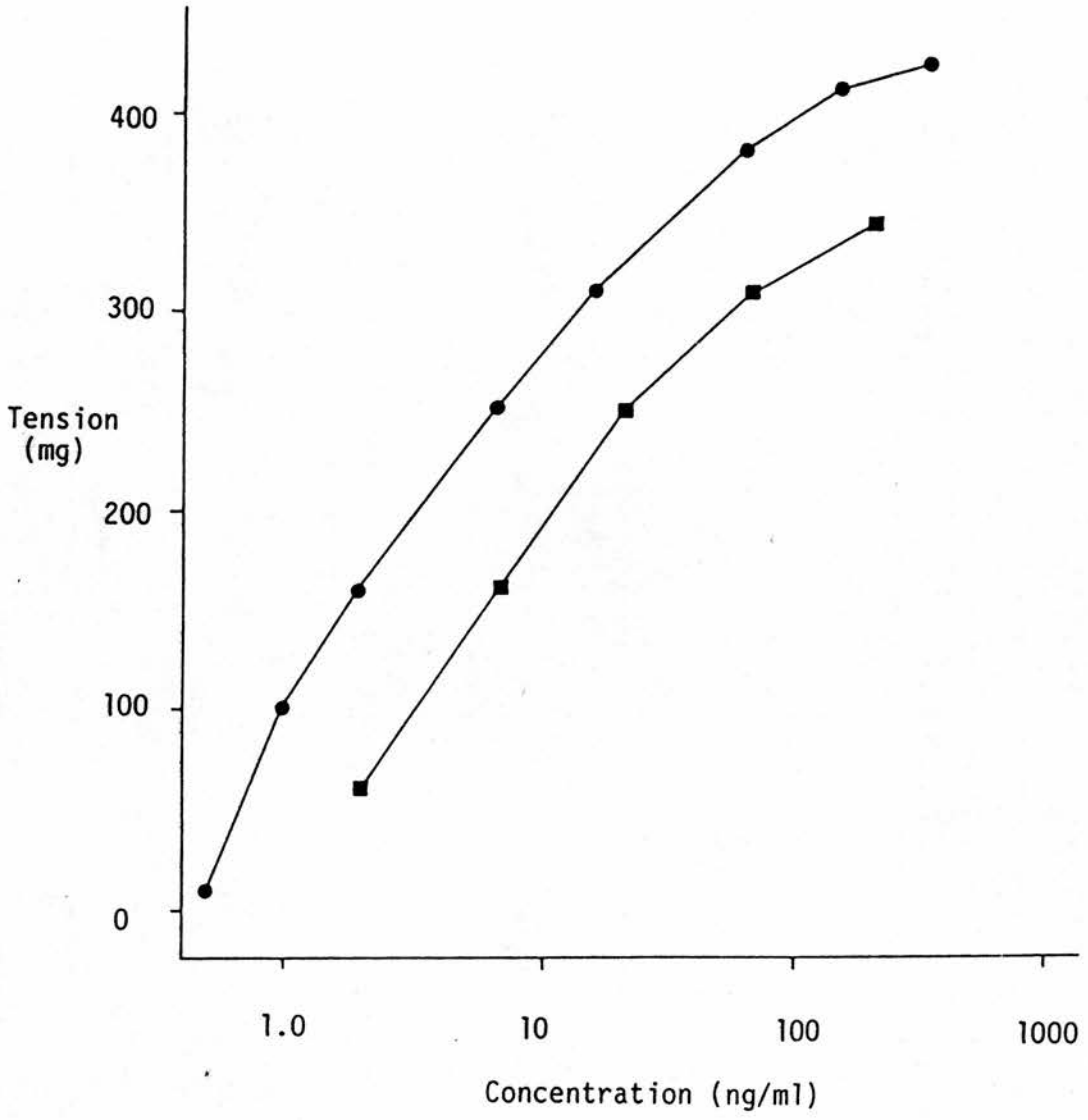


Figure E.16 Horse iris muscle preparation: cumulative concentration-response relationships for ICI 79939, $PGF_{2\alpha}$ (solid circle) and $PGF_{2\alpha}$ (solid square).

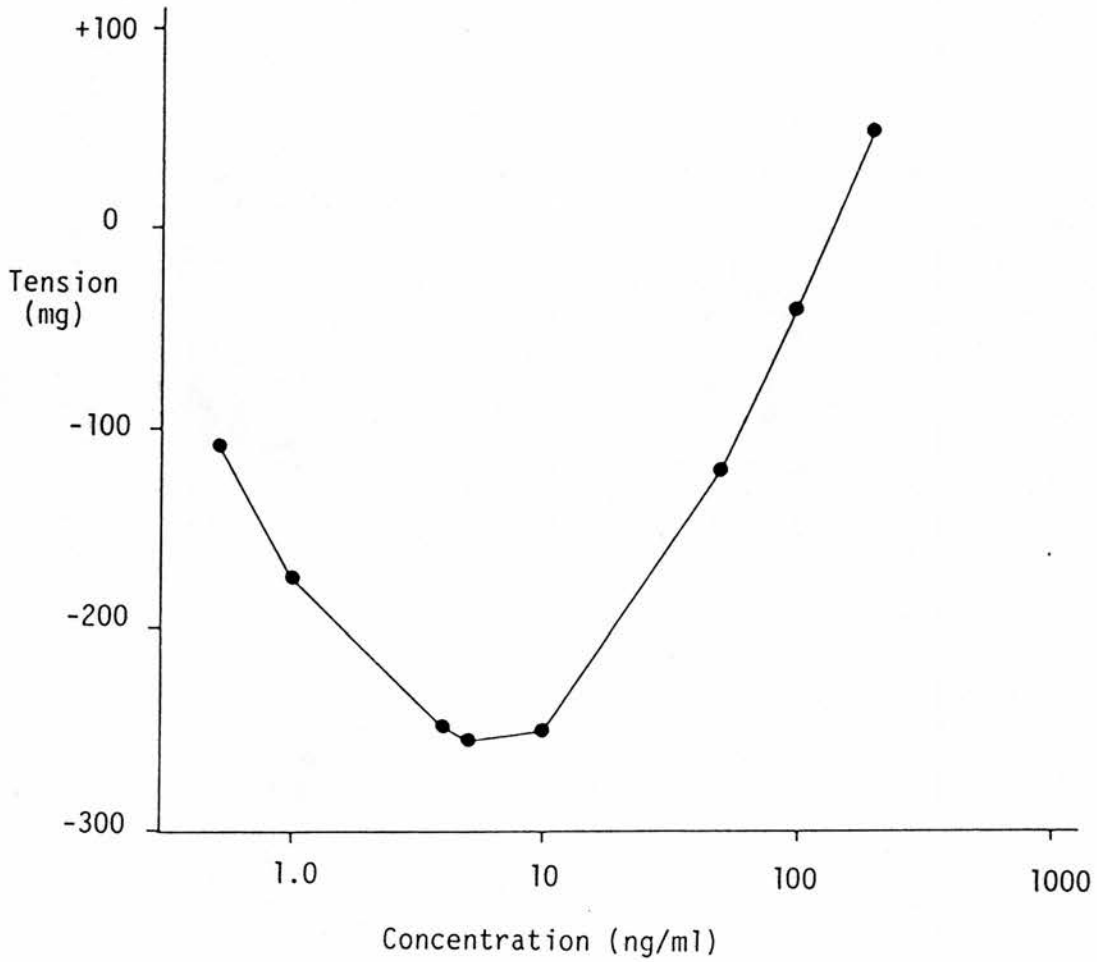


Figure E.17 Horse iris sphincter preparation: cumulative concentration-response relationship for PGE_2.

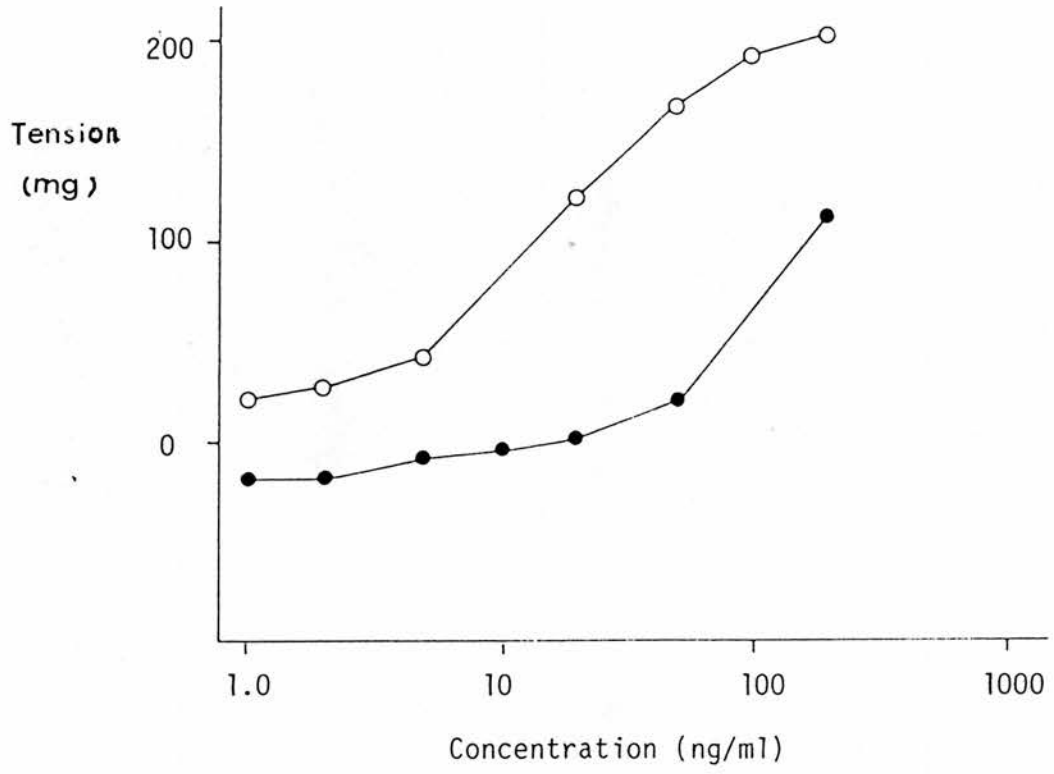


Figure E.18a Horse iris sphincter muscle preparation: cumulative concentration-response relationship for $PGF_{2\alpha}$ (open circle) and PGE_2 (solid circle).

response much lower than that obtained with PGF2a (range 10-30%) (Figure E.18b) and PGE2. EC50 for 16.16-dimethyl PGE2 was 4.1 ng/ml (range 2.0-7.2. n=6).

As expected, ICI 80205 was fairly active on the preparation. Its log dose-response curve was steeper than that of PGF2a, and its maximum response was higher than that of PGF2a.

PGE1 had more potent relaxant effect than PGE2 on the horse iris sphincter preparation (see Table E.10). For the contractile effect PGE1 was less active than PGE2. Against the background of PGE1(2.7 ng/ml)-induced maximum relaxation, PGE2 did not produce any further relaxation and at concentrations higher than 10 ng/ml it elicited a contractile effect.

Both ZK 36374 and PGI2 produced relaxant responses at concentrations of 10-500 ng/ml and at higher concentrations of both ZK 36374 and PGI2 showed contractile activities. When PGE2 was added at a concentration which produced its own maximum relaxant effect, addition of ZK 36374 caused only a contractile effect (Figure E.19), and vice versa.

Interaction between PGE2 and PGF2a Analogues

The interactions between PGF2a and PGE2 analogues were investigated. In the presence of a fixed concentration of 16,16-dimethyl PGE2 which produced its own maximum contractile response, PGF2a caused further contraction, and vice versa (Figure E.20a and 20b). PGE2 also showed additive effects with PGF2a and ICI 81008 (Figure E.21a and 21b), while in the presence of a fixed concentration of

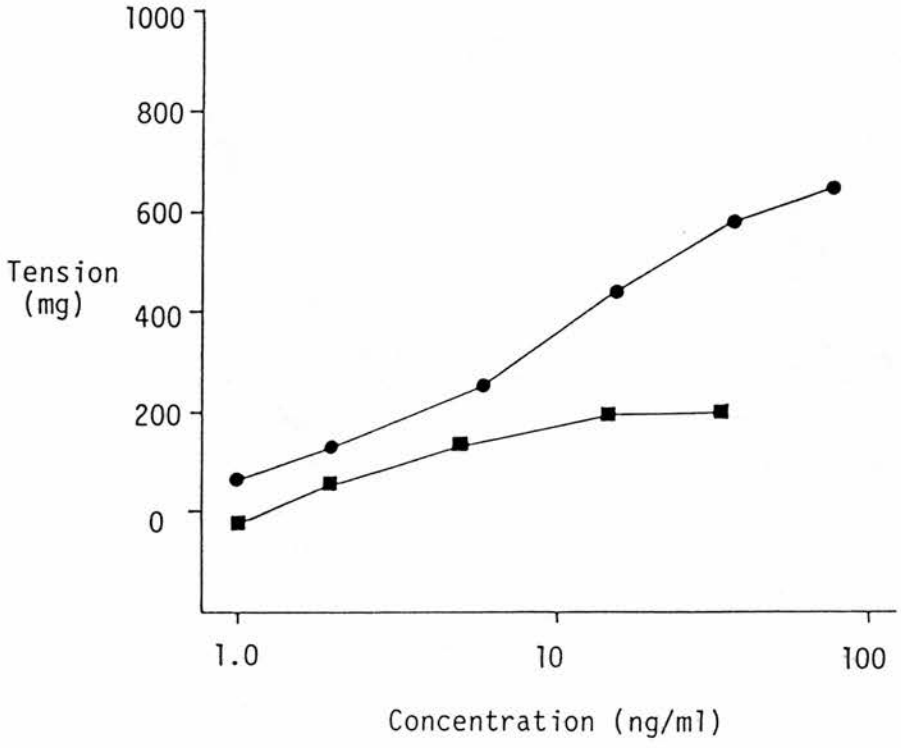


Figure E.18b Horse iris sphincter muscle preparation: cumulative concentration-response relationships for $PGF_{2\alpha}$(solid circle) and 16,16-dimethyl PGE_2 (solid square).

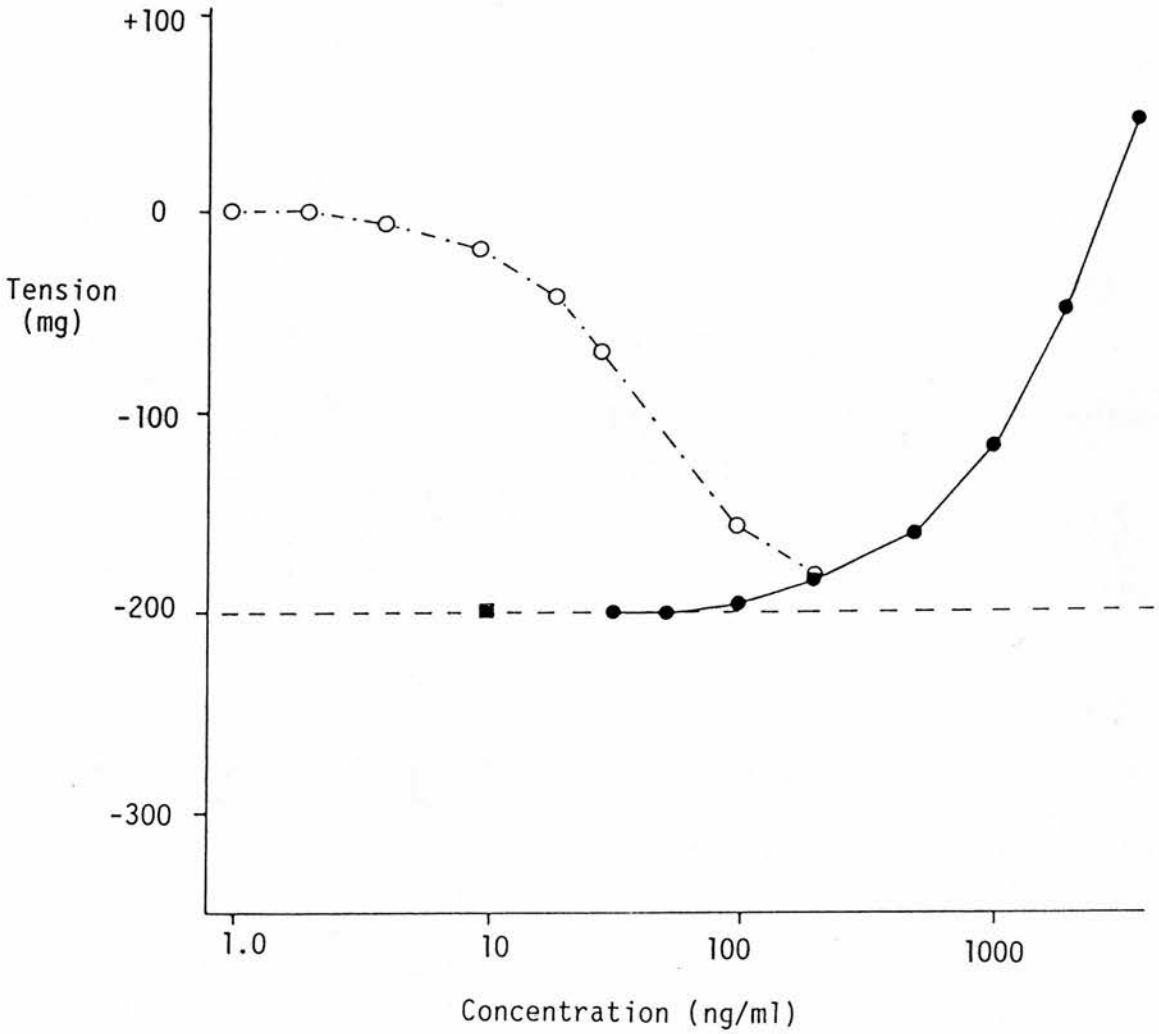


Figure E.19 Horse iris sphincter preparation: log concentration-response curve for ZK 36374 acting alone (open circle) and log concentration response curve for ZK 36374 (solid circle) in the presence of PGE_2 10 ng/ml (solid square).

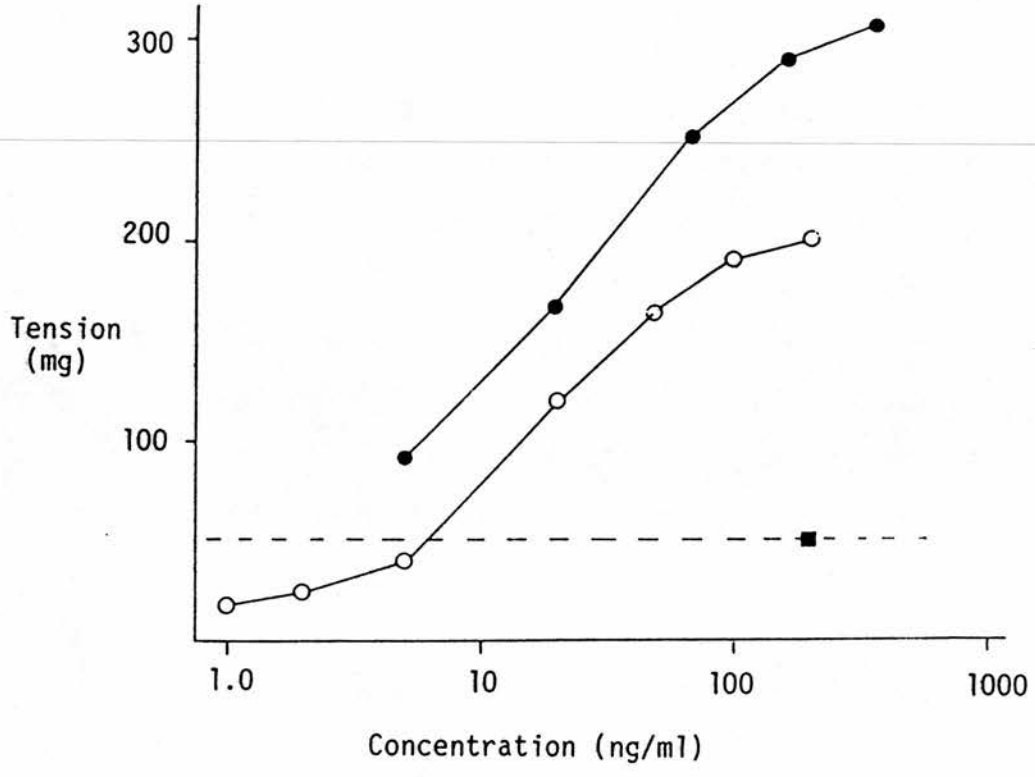


Figure E.20a Horse iris sphincter preparation: interaction of 16,16-dimethyl PGE_2 with $PGF_{2\alpha}$; log concentration-response curve for $PGF_{2\alpha}$ acting alone (open circle) and the corresponding curve (solid circle) in the presence of 200 ng/ml 16,16-dimethyl PGE_2 (solid square).

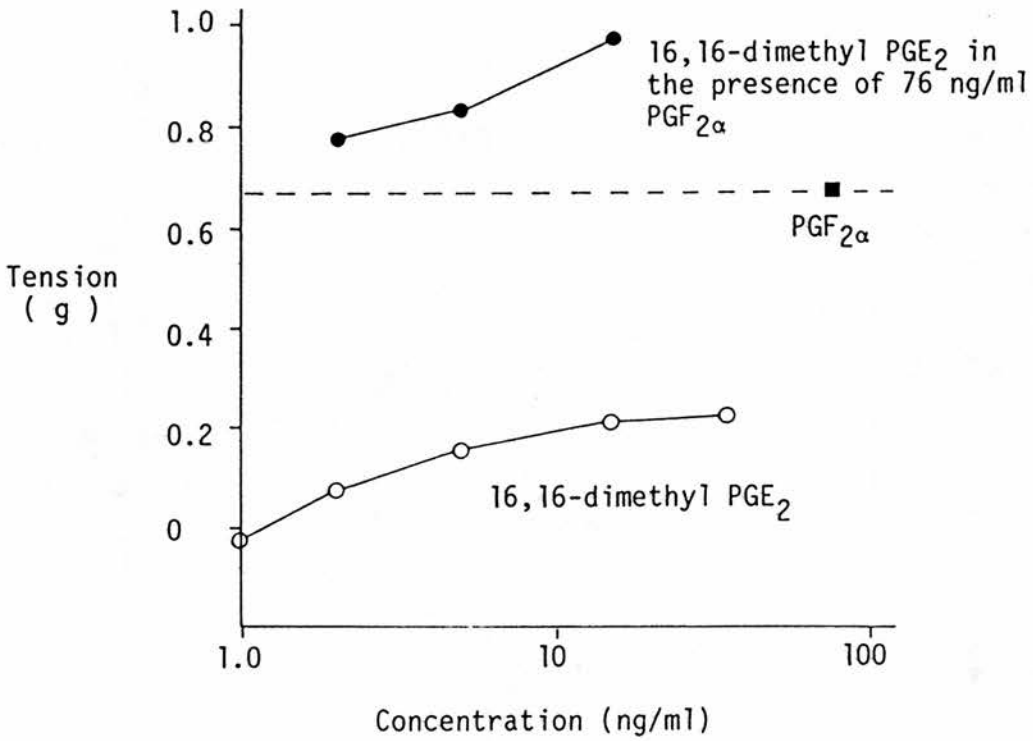


Figure E.20b Horse iris sphincter muscle: interaction of $PGF_{2\alpha}$ with 16,16-dimethyl PGE_2. Log concentration-response curve for 16,16-dimethyl PGE_2 alone (open circle) and the corresponding curve (solid circle) in the presence of 76 ng/ml $PGF_{2\alpha}$ (solid square).

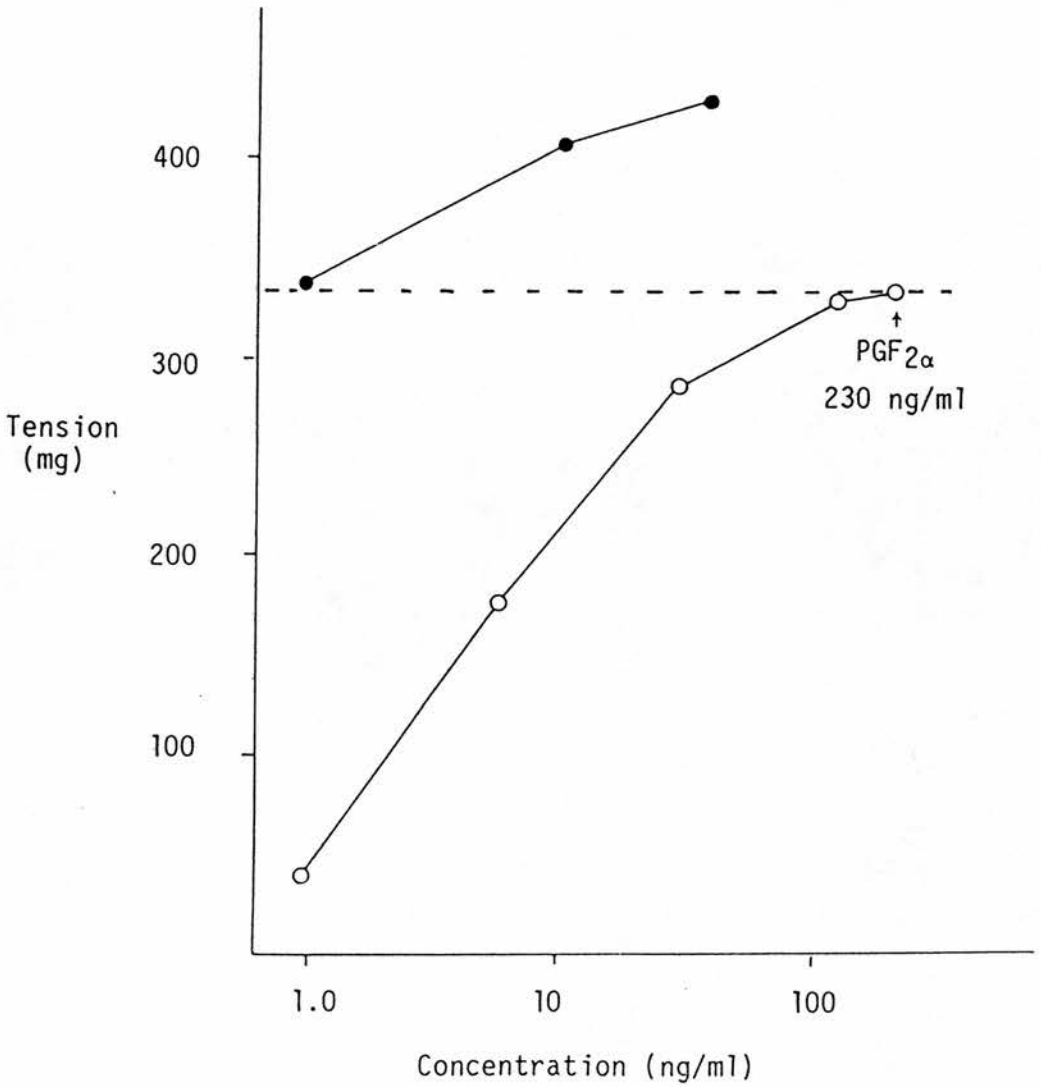


Figure E.21a Horse iris sphincter muscle: interaction of $PGF_{2\alpha}$ with PGE_2; log concentration-response curve for $PGF_{2\alpha}$ acting alone (open circle) and the curve for PGE_2 (solid circle) in the presence of $PGF_{2\alpha}$ 230 ng/ml. It shows the additive interaction of PGE_2 with $PGF_{2\alpha}$.

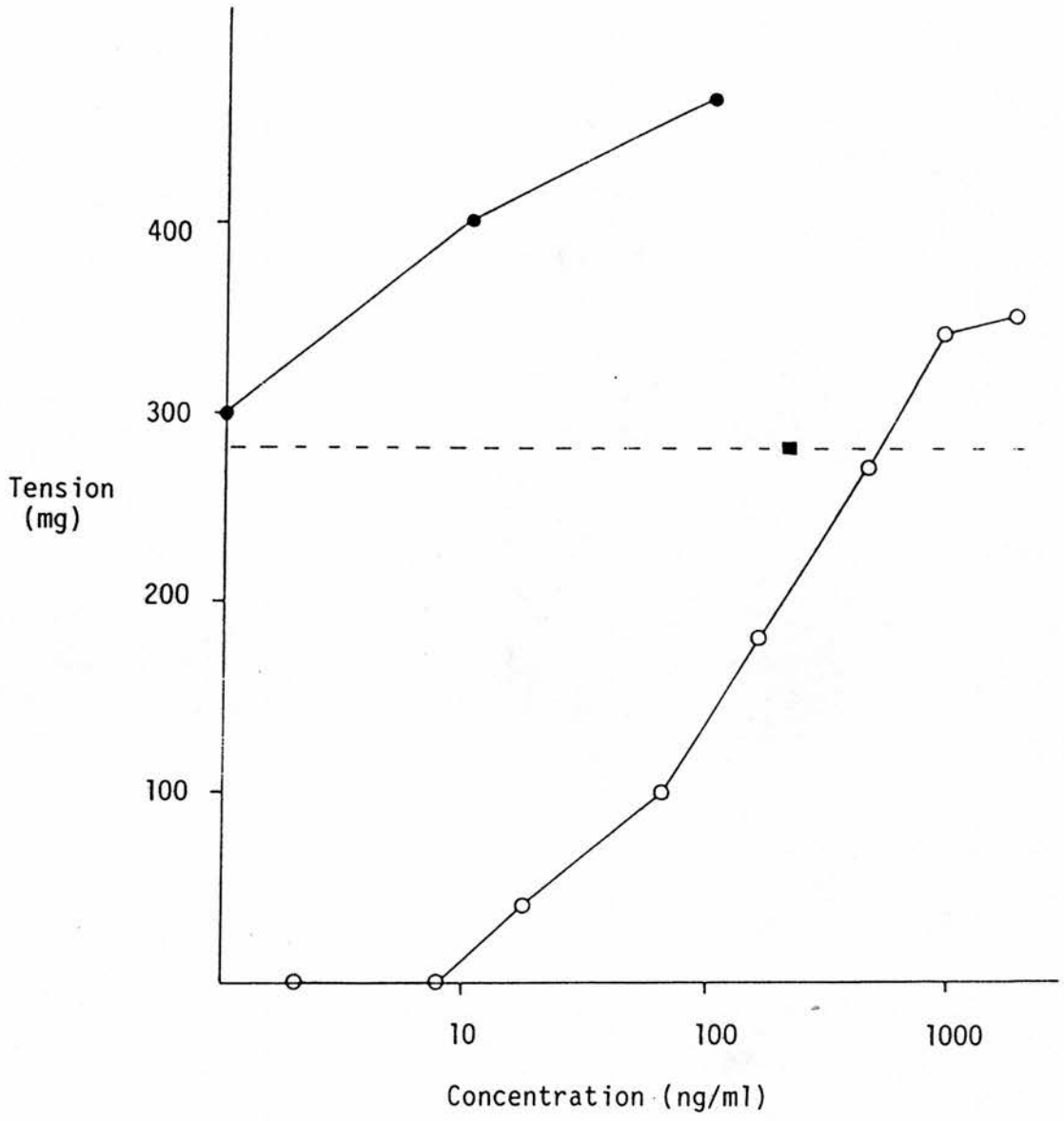


Figure E.21b Horse iris sphincter muscle: interaction of ICI 81008 with PGE_2; log concentration-response curve for PGE_2 acting alone (open circle) and the corresponding curve (solid circle) in the presence of 220 ng/ml ICI 81008 (solid square).

ICI 79939 PGF2a which produced a maximum response, PGE2 caused no further contraction.

Reversal of PGE2 Relaxant Effect by Other Prostanoids

As mentioned before, PGE2 possesses relaxant activity at low concentrations on the horse iris sphincter preparation. An initial effort was made to raise the basal tone with other contractile agents in order to study the PGE2 relaxant effect in detail. Interestingly, the inhibitory effect of PGE2 was reversed with increasing concentrations of the contractile agents. Addition of a small dose (less than 10 ng/ml) of PGE2 to the organ bath following establishment of a maximum response to PGF2a, a 60% maximum response to carbachol or 25 ng/ml 15-oxo EP 011 caused a dose-dependent contraction. One of these results are shown in Figure E.22).

Adrenaline produced an inhibitory effect on this preparation. 15-oxo EP 011 (100 ng/ml) caused a shift to the right of the dose-response curve of adrenaline.

TxA2-mimetics

15-Oxo EP 011, 15 α EP 130, 15 β EP 130, 9,11-azo PGH2 and 11,9-epoxymethano PGH2 were also tested on the horse iris sphincter preparation. All of them caused contractile effect and displayed slow on-set. Responses to 15-oxo EP 011 varied from preparation to preparation. 15-Oxo EP 011 at a fixed concentration (150 ng/ml) produced responses 13-70% of PGF2a maximum. In 2 out of 14 preparations 15-oxo EP 011 at 150 ng/ml reached its own maximum, which was 13 and 33% of PGF2a maximum. In the cases where 15-oxo EP 011 at 150 ng/ml

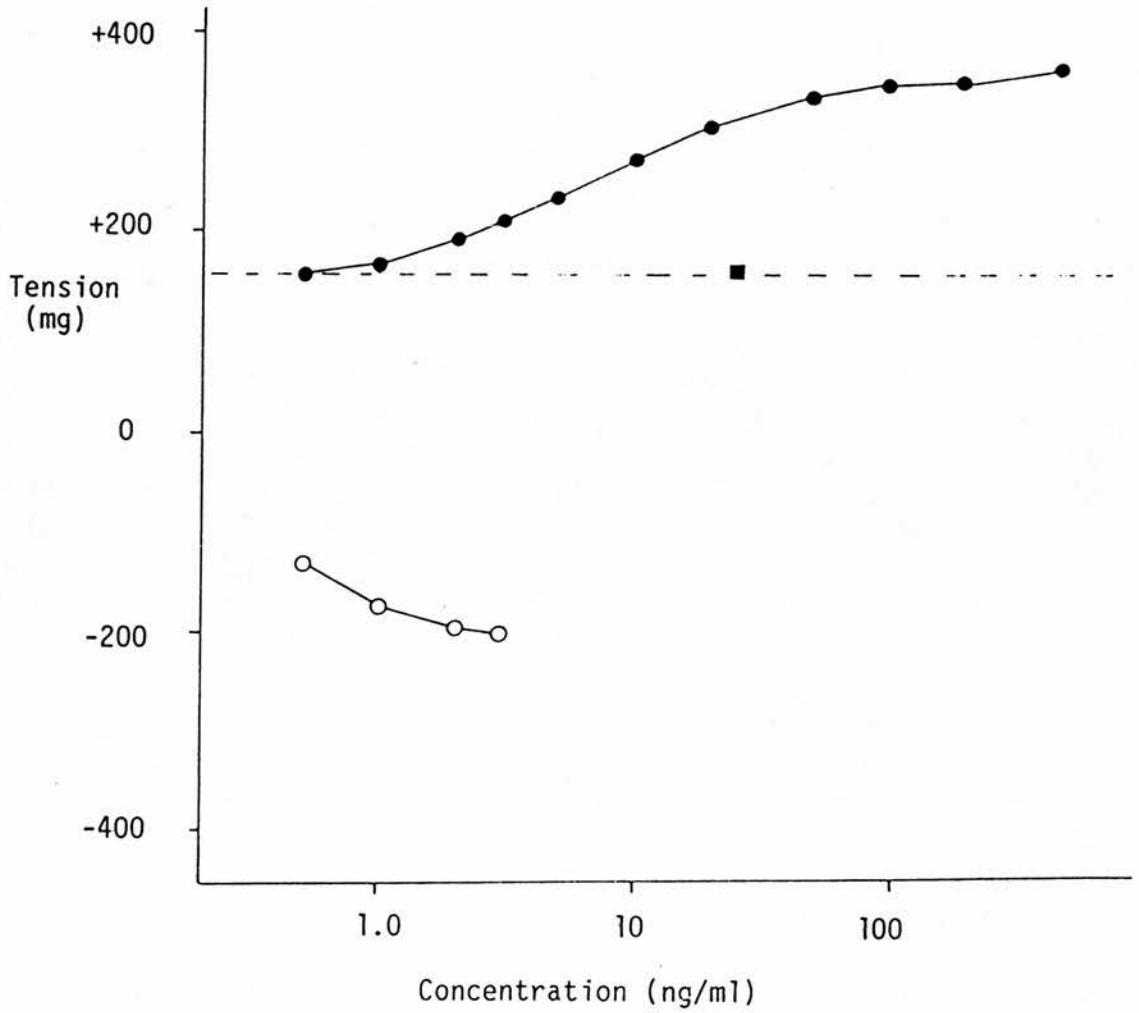


Figure E.22 Horse iris sphincter preparation: reversal of PGE_2 relaxant effect by 15-oxo EP 011; log concentration-response curve for PGE_2 alone (open circle) and the corresponding curve for PGE_2 (solid circle) in the presence of 25 ng/ml 15-oxo EP 011 (solid square).

gave a response less than 40% of PGF2a maximum, 15-oxo EP Ø11 opposed the contractile action of PGF2a (Figure E.23a) while in the cases where 15-oxo EP Ø11 at 300 ng/ml caused a response more than 60% of PGF2a maximum the interaction of 15-oxo EP Ø11 with PGF2a appeared to be additive (Figure E.23b), but after wash-out re-establishment of PGF2a dose-response relationship revealed that sensitivity to PGF2a of the preparation was markedly reduced (Figure E.24).

15 α EP 130 was more potent than 15 β EP 130. 15 α EP 130 at 500 ng/ml which gave responses 50-70% of PGF2a maximum and 15 β EP 130 at 2-5 µg/ml which gave responses 10-35% of PGF2a maximum opposed the contractile effect of PGF2a (Figure E.25a and 25b). There was loss of sensitivity to PGF2a after treatment with those two compounds (Figure E.26).

11,9-Epoxymethano PGH2 and 9,11-azo PGH2 were very weak contractile agents on the horse iris sphincter muscle preparation (see Table E.10, p51).

Rabbit Iris Sphincter Muscle

PGI2 was a potent contractile agent on the rabbit iris sphincter muscle. Responses to PGI2 reached a plateau after 2-4 min contact. The sensitivity of this preparation to PGI2 showed considerable variation; the lower and upper threshold concentrations which gave tension change of about 20 mg were 5 and 200 ng/ml. respectively. Individual values

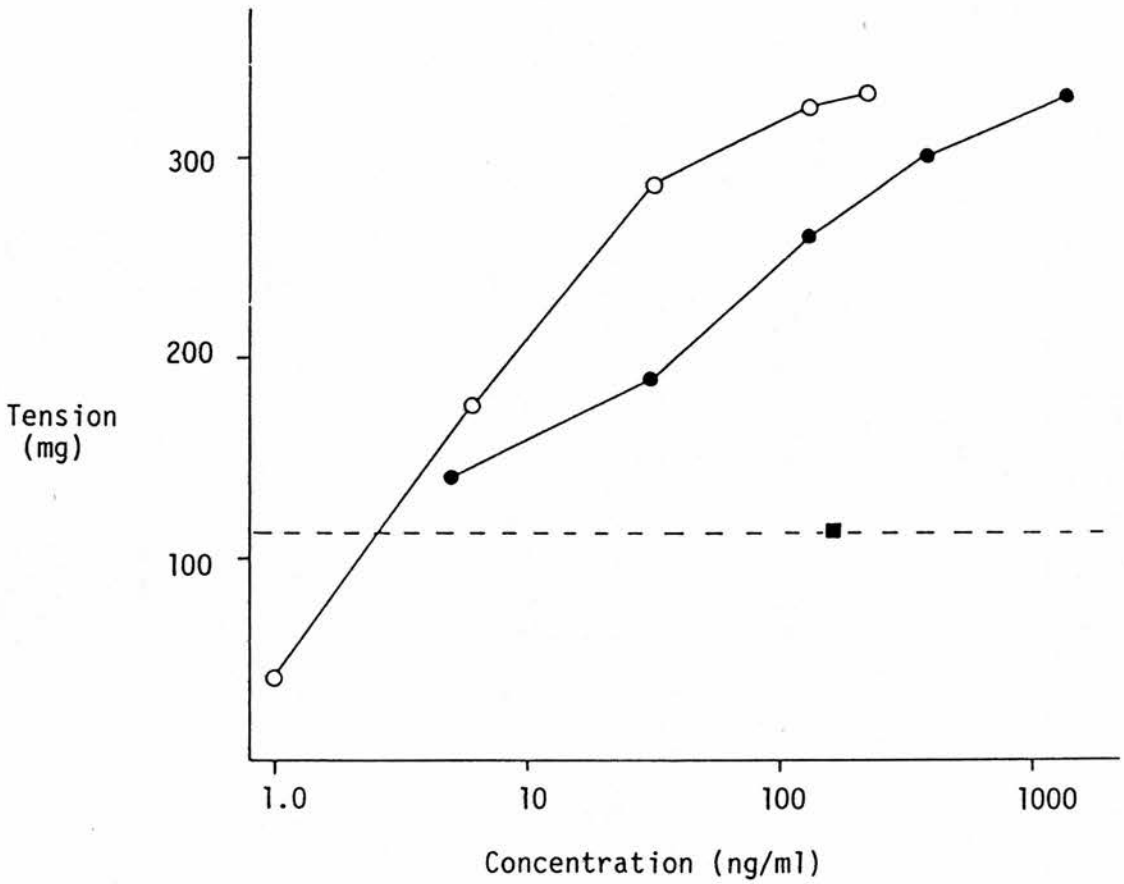


Figure E.23a Horse iris sphincter preparation: interaction of 15-oxo EP 011 with $PGF_{2\alpha}$; log concentration-response curve for $PGF_{2\alpha}$ acting alone (open circle) and the corresponding curve (solid circle) in the presence of 150 ng/ml 15-oxo EP 011 (solid square).

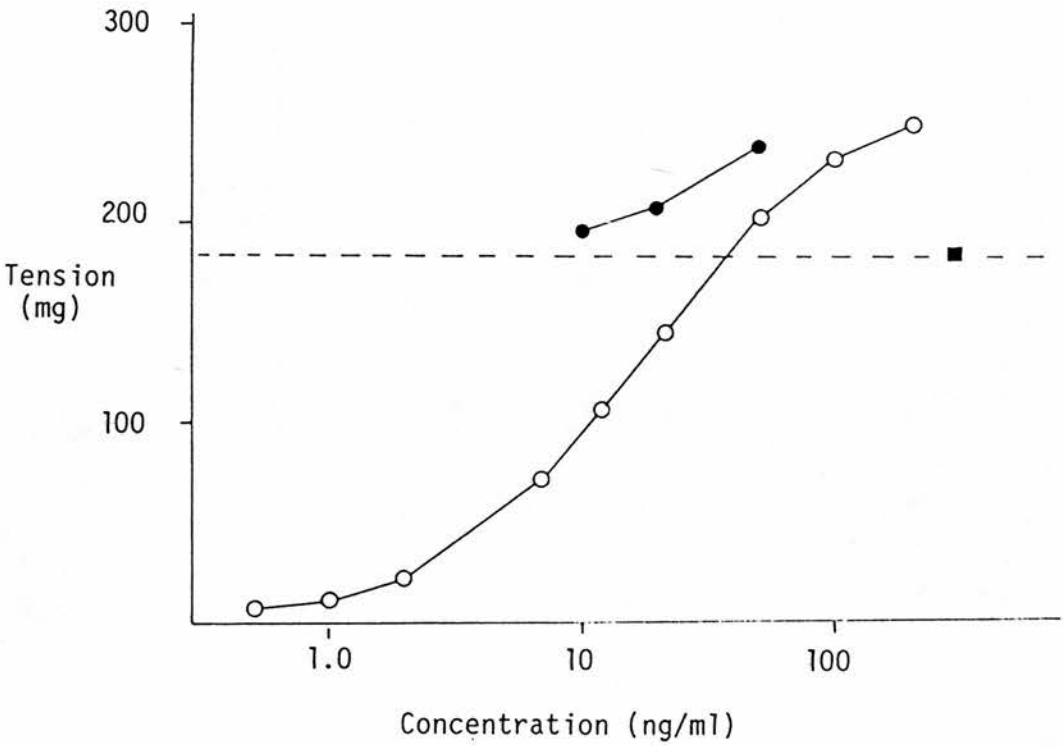


Figure E.23b Horse iris sphincter preparation: interaction of 15-oxo EP 011 with $PGF_{2\alpha}$. Log concentration-response curve for $PGF_{2\alpha}$ acting alone (open circle) and the corresponding curve (solid circle) in the presence of 300 ng/ml 15-oxo EP 011 (solid square).

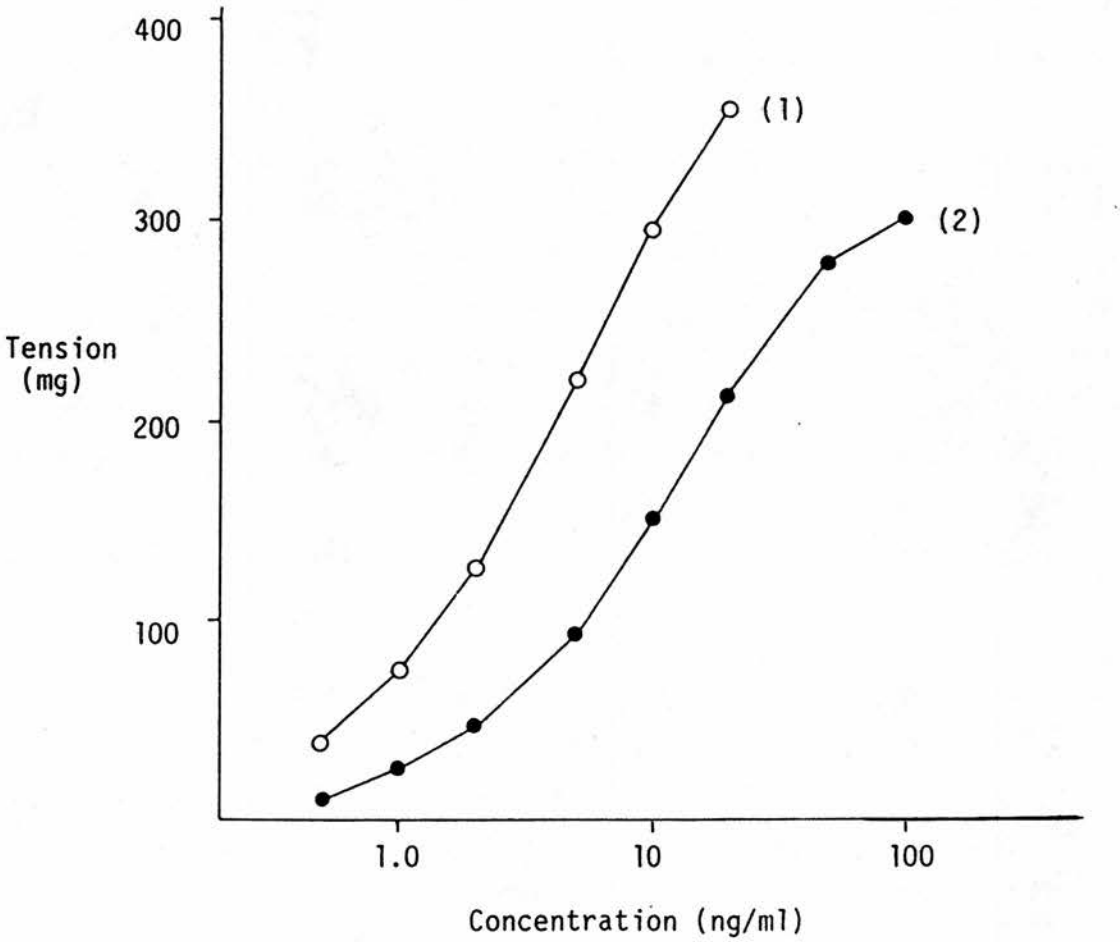


Figure E.24 Horse iris sphincter: desensitization by 15-oxo EP 011. Two cumulative concentration-response relationships for $PGF_{2\alpha}$ were established. The first sequence (1) was prior to 15-oxo EP 011 treatment, and the second (2) was after exposure to 15-oxo EP 011 150 ng/ml for 20 min and wash-out.

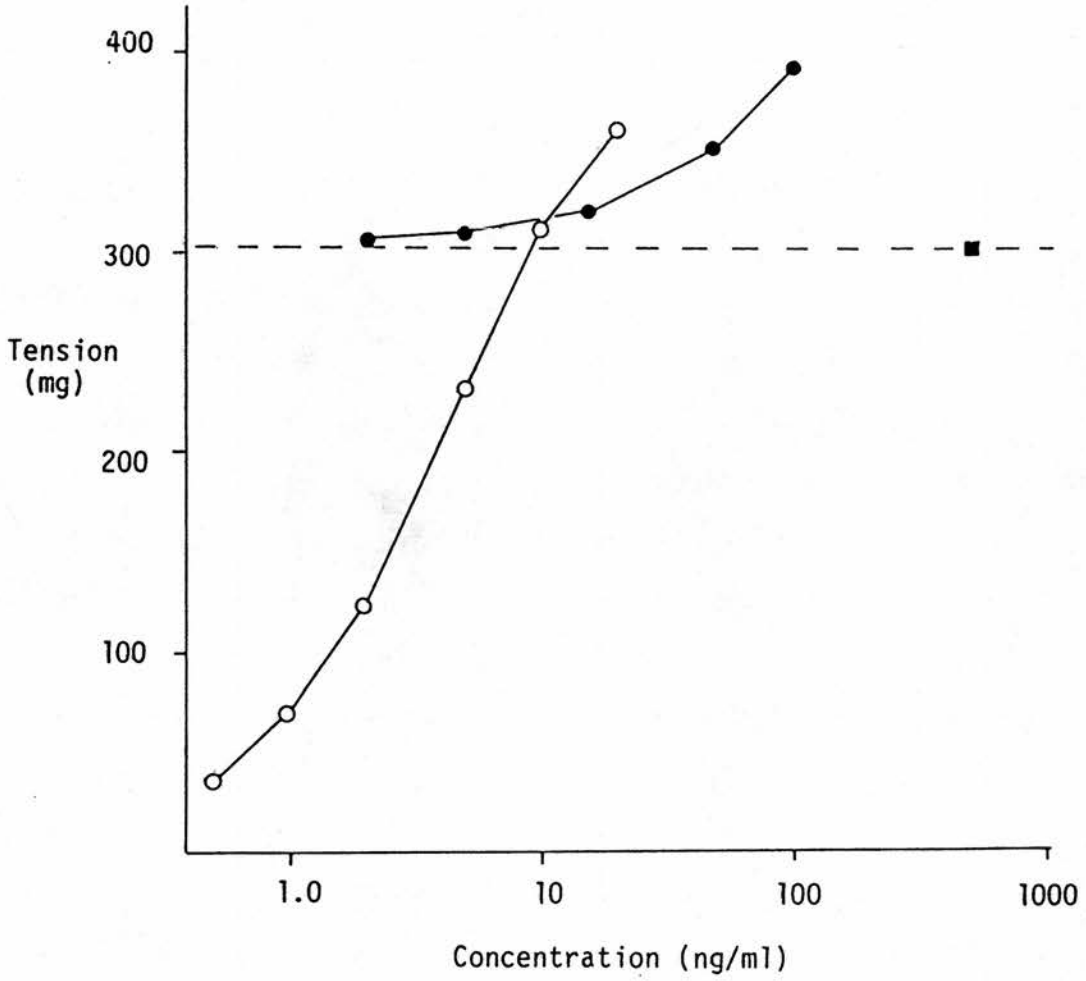


Figure E.25a Horse iris sphincter preparation: log concentration-response curve for $PGF_{2\alpha}$ acting alone (open circle) and the corresponding curve (solid circle) in the presence of 500 ng/ml 15α EP 130 (solid square).

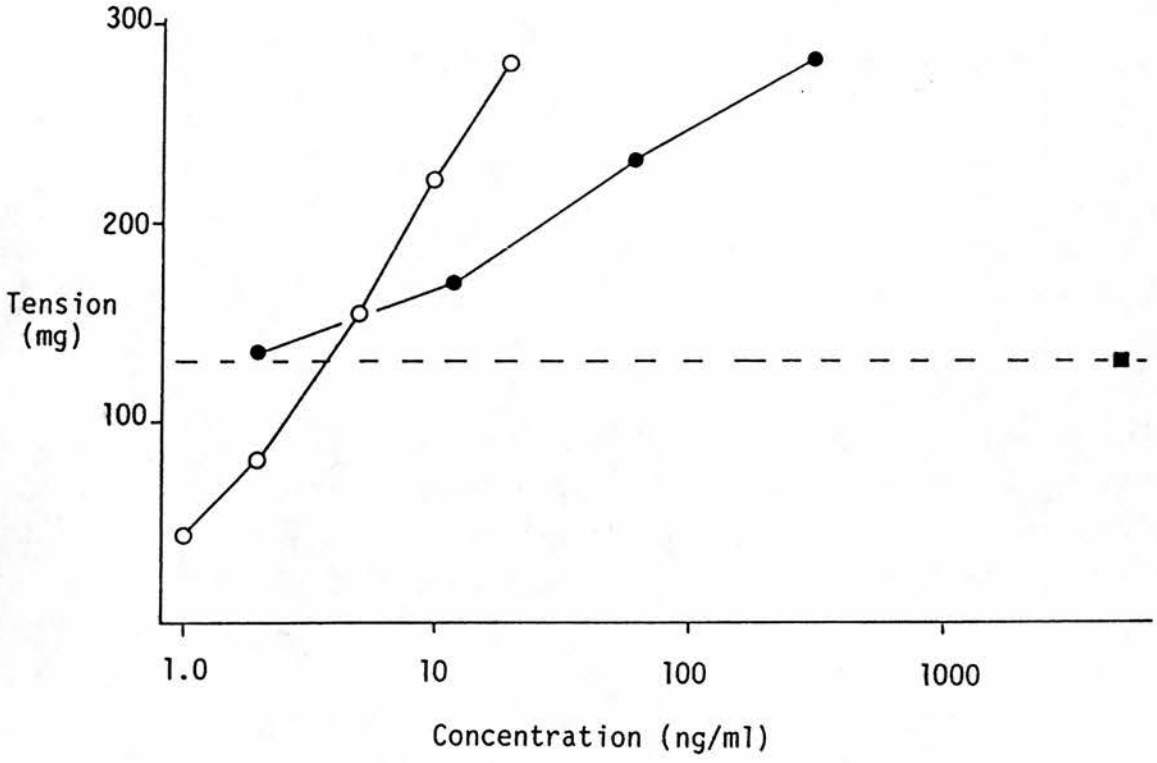


Figure E.25b Horse iris sphincter preparation: log concentration-response curve for $PGF_{2\alpha}$ acting alone (open circle) and the corresponding curve (solid circle) in the presence of 5 μg/ml 15β EP 130 (solid square).

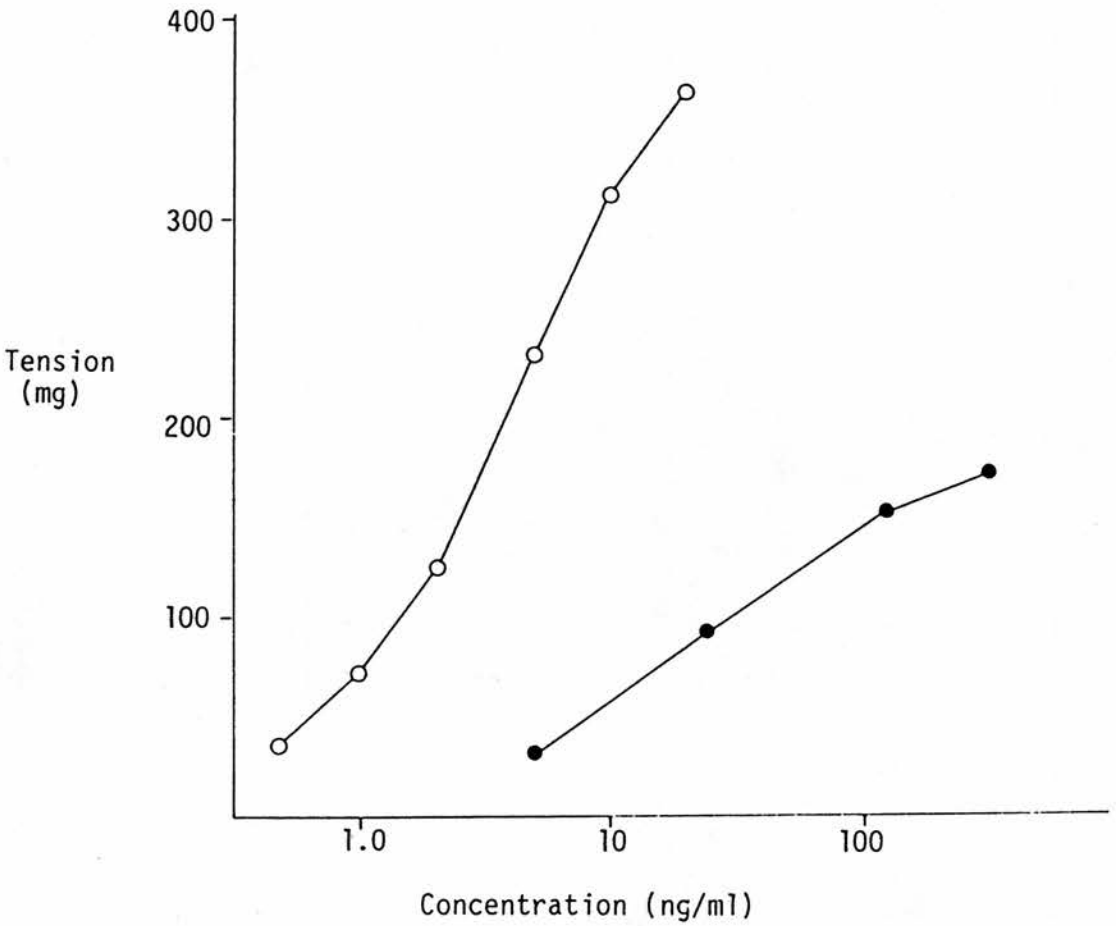


Figure E.26 Horse iris sphincter preparation: desensitization by 15α EP 130. Two sequences of cumulative concentration-response relationship were established to $PGF_{2\alpha}$ in a single preparation. The first sequence (open circle) was set up before 15α EP 130 treatment, and the second (solid circle) was set up after exposure to 15α EP 130 500 ng/ml for 40 min. and wash-out.

the
of threshold concentrationwere 5.0, 7.0, 10, 12. 13, 14, 16,
ng/ml.
16, 20, 20, 30, 34. 40. 200 EC50 values lay between 20-600 ng/ml (individual values of EC50: 20, 36, 45, 45, 59, 60, 75, 85, 120, 150, 260, 400, 410, 600). ZK 36374, a PGI2 analogue, also showed contractile activity. The equipotent molar ratio for ZK 36374 (PGI2=1.0) was 1.1 ± s.e.0.18. n=6 (individual values of EPMR: 0.59, 0.74, 0.94. 1.1, 1.6, 1.7). High concentrations of PGI2 were found to desensitise the preparation. However. the sensitivity to PGI2 was restored within 30 min of wash-out. After ZK 36374 treatment, it took a much longer time for the tissue to recover, so a cumulative concentration-response relationship for PGI2 was always established first on each preparation.

PGE2 produced a relaxant effect on this preparation. The basal tone of the preparation was, however, so low that the PGE2 relaxant effect could not be easily studied. PGI2, ZK 36374 and carbachol were therefore used to provide tone. The EC50 for the PGE2 relaxant effect was 5-1000 ng/ml. In the same preparation it did not make any difference to thePGE2 response whether PGI2, ZK 36374 or carbachol was used to provide the tone. PGE2 at concentrations up to 2 µg/ml showed no contractile activity.

PGE1 at low concentrations produced a small relaxant effect. As concentrations of PGE1 increased, a contractile effect was seen. PGE1 gave a maximum contractile response lower than that obtained with PGI2 (Figure E.27). On six preparations the values was 13, 30, 35, 56, 57 and 63% of PGI2 maximum response. EC50 values for the PGE1 contractile

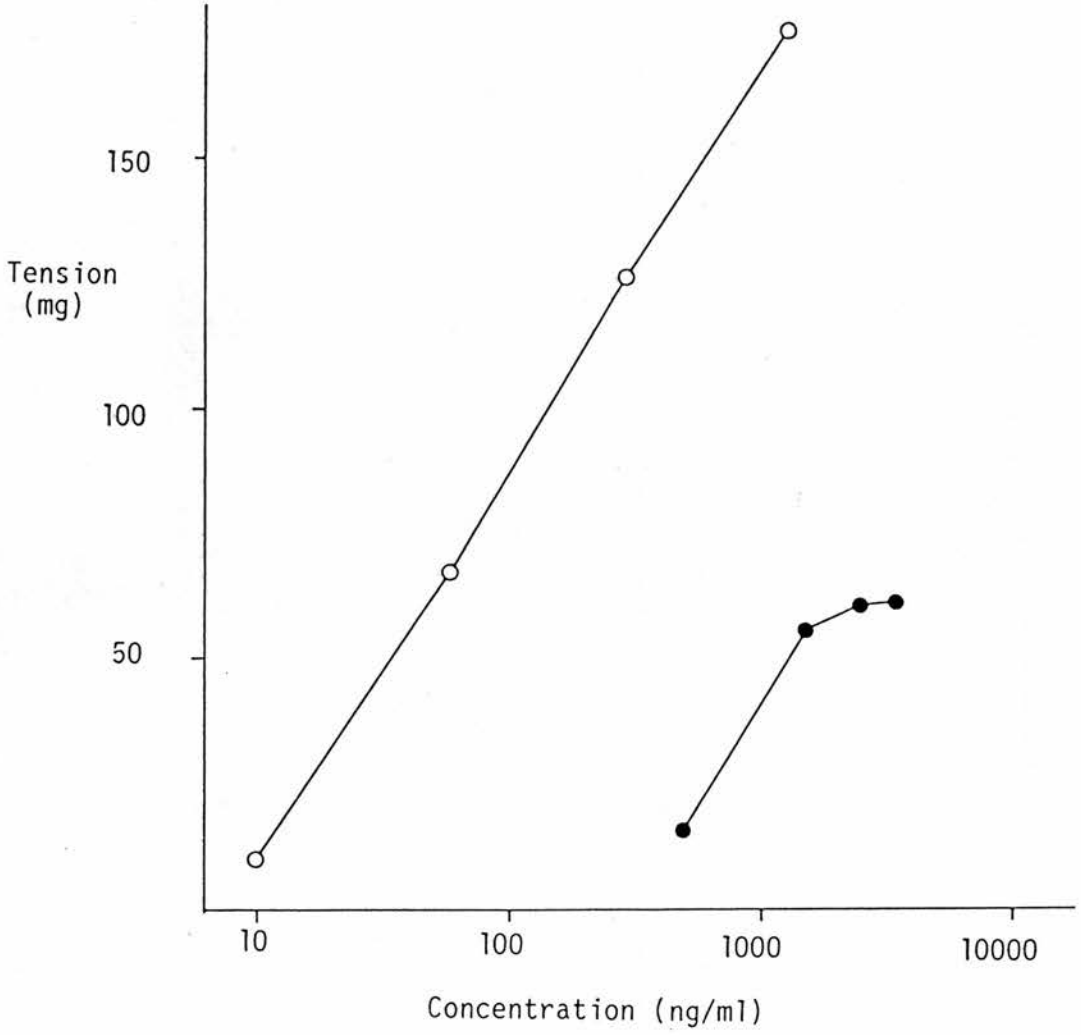


Figure E.27 Rabbit iris sphincter preparation: log concentration-response curves for PGI_2 (open circle) and PGE_1 (solid circle).

effect were 50, 75, 140, 400, 500 and 1000 ng/ml.

The interaction of PGE1 and PGE2 with PGI2 was also studied, by adding cumulative doses of PGI2 to the organ bath in the presence of a fixed concentration of PGE1 or PGE2. PGE1 opposed the contractile action of PGI2. A typical result is shown in Figure E.28. After PGE1 treatment, following wash-out, the tissue showed loss of sensitivity to PGI2. The influence of PGE2 on PGI2 action depended on the tissue sensitivity to PGE2. On the preparation which was sensitive to PGE2, the effect of PGI2 was depressed in the presence of PGE2, while on a preparation less sensitive to PGE2, PGE2 had little effect on the response to PGI2.

11,9-epoxymethano PGH2, PGD2, PGI1 and PGF2a were very weak on this preparation. These above compounds at 100 ng/ml produced no effect.

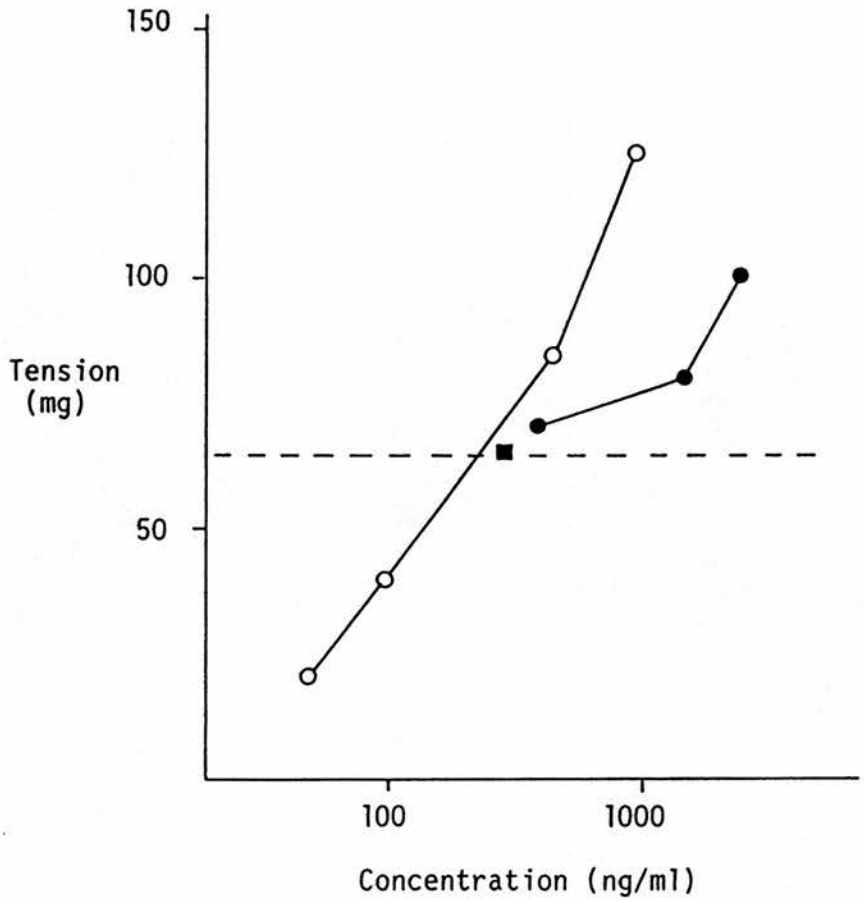


Figure E.28 Rabbit iris sphincter preparation: interaction of PGE_1 with PGI_2; log concentration-response curve for PGI_2 acting alone (open circle) and the corresponding curve (solid circle) in the presence of 300 ng/ml PGE_1 (solid square).

DISCUSSION

The possible prostaglandin receptor types together with the direction of response found in the different iris preparations are summarised in Table E.11.

It is obvious from what has been said in the results section that information obtained on tissues in which relaxant effects are absent, is more easily analysed in terms of receptor classification.

Dog and Cat Iris Sphincter Muscles

The dog and cat iris sphincters appear to be the simplest of the systems studied. Our results suggest the presence of a single receptor type at which PGF2a and its closely related analogues are the most active contractile agonists, whereas PGE2, PGI2 and 11.9-epoxymethano PGH2 show only weak activity. This accords with work by Kennedy, Coleman, Humphrey, Levy & Lumley (1982). The very low activity of 16,16-dimethyl PGE2 is in complete contrast to its high activity on the bovine iris sphincter. ZK 36374 is also a weak contractile agent and does not oppose the action of PGF2a. These results may indicate that a PGE-sensitive system similar to that found in the bullock iris sphincter is not present in the dog iris sphincter. It is possible that the activity of PGE2 is due to its ability to interact with the PGF2a receptor.

Table E.11 Summarization of receptor types and the types of mechanical responses in the iris sphincter preparations from different animals.

	Iris Sphincter				
Receptor	Bullock	Dog	Cat	Horse	Rabbit
PGD_2					
PGE_2	+↑			+↑	
				+↓	+↓
$PGF_{2\alpha}$		+↑	+↑	+↑	
PGI_2				+(?)↓	+↑
TxA_2	+↑				

+ indicates definite pharmacological evidence for existence of receptor
↑ or ↓ indicates contractile or relaxant effect associated with agonist action at receptor.

ICI 81008 is a potent luteolytic agent (Dukes, Ressell & Walpole, 1974), mimicking the action of PGF2a. It shows similar activity to PGF2a on the rabbit jejunum in vitro and the rabbit oviduct in vivo (Welburn & Jones. 1978). Its low activity on PGE-sensitive preparations has been documented previously ---- guinea-pig uterus in vitro (Dukes, Russell & Walpole, 1974) and guinea-pig ileum in vitro (Welburn & Jones, 1978). ICI 81008 therefore seems to be a specific full agonist on the PGF2a receptor.

ICI 79939 PGF2a is another potent PGF2a mimetic, but it lacks selectivity. In addition to the PGF2a receptor it activates the PGE2 receptor on the bullock iris sphincter preparation, which we will mention later, and the TxA2 receptor on human platelets and rabbit aortic strips (Jones, Wilson & Marr, 1979; Jones, Peesapati & Wilson, 1982).

EP 045, EP 092 and EP 116 do not block the activity of PGF2a on the dog iris sphincter. These compounds effectively and specifically block responses to 11,9-epoxymethano PGH2 on preparations sensitive to TxA2. Their affinity constants are listed in Table Table E.12. Contraction of the dog iris sphincter induced by 11,9-epoxymethano PGH2 is not blocked by EP 045. This suggests that the weak contractile activity of 11 9-epoxymethano PGH2 is due to an interaction with the PGF2a receptor site.

Two PGE2 analogues, ICI 80205 and ICI 79939 PGE2 show surprisingly high PGF2a-like activity (see Table E.6,P39). However. they both show considerable TxA2-like activity as well (Jones, Peesapati & Wilson, 1982). It therefore appears

Table E.12 Affinity constants for EP 045, EP 092 and EP 116 on several preparations.

Tissue	EP 045	EP 092	EP 116
	(Affinity Constants M^{-1})		
*Rabbit aorta	2.0×10^6	18×10^6	10×10^6
*Dog saphenous vein	22×10^6	87×10^6	210×10^6
*Guinea-Pig trachea	33×10^6	89×10^6	580×10^6
Bullock iris sphincter	7.2×10^6	280×10^6	780×10^6
Rat anococcygeus muscle	4.9×10^6	19×10^6	83×10^6
Rat gastric fundus	8.2×10^6		180×10^6

11,9-Epoxymethano PGH_2 was used as agonist.
Bullock iris sphincter and rat gastric fundus preparations were in Krebs solution containing 10^{-6} M indomethacin, and guinea-pig ileum was in Krebs containing 10^{-6} M indomethacin and 2×10^{-8} M atropine.
* denotes data from Jones, R.L..

that the substitution of a para-halogenated phenoxy group at the C16 position can increase agonist potency in a number of systems, but this is accompanied by a loss of specificity.

The TxA2-mimetics listed in Table E.8/4 P.44 elicited contraction of the dog iris preparation. These drugs can be divided into two groups: one group with an aromatic substituent in the ω-chain, i.e. 15-oxo EP 011, 15 α EP 130, 15 β EP 130 and 15-oxo EP 130, oppose the effect of PGF2a, and the other without the aromatic substituent, i.e. 9,11-ethano PGH2 and 9,11-azo PGH2, fail to affect PGF2a action. Compounds in the first group probably act on PGF2a sites since EP 116 does not block 15-oxo EP 011-induced contraction, 15-oxo EP 011 desensitised preparations lose sensitivity to PGF2a, and the interaction of 15-oxo EP 011 with carbachol is additive. Their low maximum response seems to indicate that they might be partial agonists on PGF2a receptor sites. But desensitization makes it difficult to be confident that this is the case. The long-term desensitization caused by 15-oxo EP 011 is probably due to its high lipophilicity which prevents it from being washed out. 15-Oxo EP 011 also has long acting effects on thromboxane-sensitive preparations. However, this action is easier to investigate since thromboxane-sensitive systems are in general resistant to desensitisation.

The two compounds with natural ω-chains, i.e. 9,11-ethano PGH2 and 9,11-azo PGH2, are very weak on the PGF2a receptor sites and do not oppose the PGF2a effect. It is assumed that maximum response induced by a partial

agonist requires 100% occupancy of the receptor. However, if both 9,11-ethano PGH2 and 9,11-azo PGH2 have low affinity, their binding to the PGF2a receptor may be precarious and readily be displaced by PGF2a which merges firmly with the receptor. Or an alternative explanation could be that partial agonists with low affinity might not occupy all the receptors for maximum response, so that there were spare receptors for a full agonist to act on. To oppose PGF2a action much higher concentration will be required. Their contractile effect in this preparation is not due to activation of TxA2 receptors, since EP 116 did not block the contraction. And it is unlikely for them to produce contraction by non-specific activities, for example, ionophore-like activity, as their EC 50 values are around 100 ng/ml. A PGF2a receptor antagonist will be needed before further conclusions can be drawn.

One thing worth mentioning is the suggestion by Crawford, van Alphen, Cook and Lands (1978) that there is a TxA2-sensitive system in the cat iris sphincter. They found that PGH2 was as potent as PGF2a, whereas the epoxymethano analogues, 11,9-epoxymethano PGH2 and 9,11-epoxymethano PGH2 were very weak. The epoxymethano analogues did, however, inhibit the PGH2-induced contractions. They argue that since the formation of PGF2a from PGH2 is a non-enzymic process (Chan, Nagasawa, Takeguchi & Sih, 1975) and the epoxymethano analogues are known to be effective inhibitors of the conversion of PGH2 to TxA2 in platelet microsomes (Sun, 1977), then TxA2 synthetase action must mediate the contraction following PGH2 addition.

However, it has been shown that 11,9-epoxymethano PGH2 has the same profile of activity as TxA2 on a number of smooth muscle preparations (Coleman, Humphrey, Kennedy, Levy & Lumley, 1981). The low activity of 11,9-epoxymethano PGH2 on the dog and cat iris sphincter muscle and the inability of EP 045 to block the weak activity of 11,9-epoxymethano PGH2 on the PGF2a-sensitive preparation indicate that the TxA2 receptor site does not exist on these two preparations. This is supported by work from Coleman, Humphrey et al. (1981). They have demonstrated that PGH2 is also active in the dog iris sphincter, that imidazole, a TxA2 synthetase inhibitor, does not affect the PGH2 action and that generated TxA2 is a very weak agonist. Since we have shown that the dog and cat iris sphincter muscles have similar properties in terms of prostaglandin receptors, the results from the two preparations may be comparable. Further studies on the metabolism of PGH2 in these iris preparations may be useful. The 9,11-peroxy link in PGH2 is extremely easy to reduce (Stannous Chloride in ethanol/water, room temperature, 5 min), and it is possible that a rapid enzymic or non-enzymic reduction to PGF2a occurs in the tissue.

Bullock Iris Sphincter Muscle

Our results suggest that the bullock iris sphincter muscle is more complicated: it contains two different types of prostanoid receptor. One is more sensitive to PGE analogues and the other to thromboxane analogues. However, it is

amenable to analysis since we have specific TxA2 receptor antagonists available.

The PGE-sensitive system responds to PGE2 and its 16,16-dimethyl and 17,18,19,20-tetranor-16-p-chlorophenoxy analogues. The order of agonist potency on the PGE2-sensitive system is ICI 80205> 16,16-dimethyl PGE2>PGE2> PGE1> 11-deoxy PGE1> PGF2a> PGI1> PGI2> PGD2. ICI 80205 and 16,16-dimethyl PGE2 are highly active, about 5 times and 2 times more active than PGE2, respectively; PGE1 and PGF2a are less active (Table E.1, P15).

The system is further characterized by the unique action of ZK 36374. This stable analogue is structurally very close to PGI2 and shows properties similar to PGI2 on bovine coronary artery, human platelets (Schror, Darius, Matzky & Ohlendorf, 1981) and rabbit iris sphincter (see results from rabbit iris sphincter muscle in this section). Its potency is equivalent to PGI2 on the human platelets (inhibition of aggregation) and on the rabbit iris sphincter (contractile effect). On the bovine iris sphincter preparation, ZK 36374 elicits threshold contractile effects at concentrations of 1-2 ng/ml whereas PGI2 concentrations for similar responses are 10-30 times higher. Moreover, ZK 36374 seems to act as a partial agonist, giving a lower maximum than PGE2 and opposing the actions of PGE2. 16,16-dimethyl PGE2, PGF2a and PGI2, but not those of 11.9-epoxymethano PGH2 and carbachol. EP 045, a TxA2 receptor antagonist, does not block the effect of ZK 36374. Thus, ZK 36374 appears to be a partial agonist on PGE2-sensitive sites.

PGI2 and PGI1 also showed maxima lower than PGE2. That is 76% for PGI2 and 77% for PGI1. PGI1 was about twice as potent as PGI2: this is in contrast to the much greater potency of PGI2 on the rabbit iris sphincter preparation. The interaction of PGI2 or PGI1 with PGE2 has not been studied, but it may be proposed that PGI2 and PGI1 are partial agonists on PGE2-sensitive sites because of the structural similarities between PGI1, PGI2 and ZK36374, and the inability of EP 045 to block PGI2 action. However, it is possible that instability of PGI2 in aqueous solution favours the attainment of a lower maximum response.

The other prostanoid-sensitive contractile system shows properties similar to those found for the rabbit aorta and the dog saphenous vein (Jones, Wilson & Marr, 1979; Jones & Wilson, 1980; Jones, Peesapati & Wilson, 1982) (Table E.13). Thus the 11,9-epoxymethano PGH2 analogue is a potent full agonist. The results in this study have indicated that the system on which 11,9-epoxymethano PGH2 acts is a TxA2-sensitive system because the actions of both generated TxA2 and 11,9-epoxymethano PGH2 can be blocked by EP 045, and not by ZK 36374, and TxA2 is more potent than PGH2 in the presence of indomethacin. This is consistent with recent studies which have shown that 11,9-epoxymethano PGH2 has the same profile of activity as TxA2 on smooth muscle and can be considered as a selective TxA2 mimetic despite being chemically an analogue of PGH2 (Coleman, Humphrey, Kennedy, Levy & Lumley, 1981). The use of the stable thromboxane receptor agonist, 11,9-epoxymethano PGH2,

Table E.13 Activities of TxA_2 agonists on bullock iris sphincter, rabbit aorta and dog saphenous vein preparations.

Compound	Equipotent molar ratios		
	BIS	RA	DSV
11,9-epoxymethano PGH_2	1.0	1.0	1.0
9,11-azo PGH_2	0.74	1.4	1.2
(±)EP 011	0.033	0.12	0.087
PTA_2	240 nM 47%	210 nM 47-60%	21 nM 24-30%

BIS, RA and DSV indicate bullock iris sphincter, rabbit aorta and dog saphenous vein, respectively.
PTA_2 is a partial agonist in all the preparations. Lower and upper values of the relative maximum response (11,9-epoxymethano PGH_2=100%) together with the concentration of the partial agonist required to produce a response 50% of its own maximum are given.

considerably simplifies the investigation of compounds with thromboxane blocking activity. The action of the 11,9-epoxymethano PGH2 analogue is blocked by the semicarbazone analogues, EP 045 and EP 116. The affinity constant for EP 045 on the bovine iris sphincter (7.2×10^6 M^{-1}) is less than those found previously for the 11.9-epoxymethano PGH2 analogue/EP 045 combination on the dog saphenous vein (2.2×10^7 M^{-1}) and guinea-pig trachea (3.3×10^7 M^{-1}). However. recently Jones (Personal Communication) has found that the affinity constant for a new batch of EP 045 on the bovine iris sphincter is 4.6×10^7 M^{-1}. EP 116 is about 100 times more potent than EP 045 in blocking the action of the 11.9-epoxymethano PGH2 analogue on the bovine iris sphincter preparation.

The rank order of potency for the TxA2 mimetics is EP 011 $\geq$ 15-oxo EP 011> 9,11-azo PGH2 $\geq$ CTA2> 11,9-epoxymethano PGH2. Table E.2 P22 The two 16-p-fluorophenoxy compounds are considerably more powerful agonists than 11.9-epoxymethano PGH2 (factor of about 30), and their threshold concentration is about 0.1 nM. 9,11-Azo PGH2 and CTA2 have activities similar to 11.9-epoxymethano PGH2. EP 011, 15-oxo EP 011 and CTA2 exhibit a slow on-set and prolonged duration of action: this profile of action has been observed on all thromboxane-sensitive smooth muscle preparations examined to date. The 9,11-ethano PGH2 and the pinane TxA2 analogue are partial agonists.

Weak thromboxane-like activity on smooth muscle has been previously demonstrated for 16,16-dimethyl PGE2 (Jones &

Wilson, 1980). On the rabbit aorta and dog saphenous vein, 16,16-dimethyl PGE2 is 33 and 55 times respectively less active than 11,9-epoxymethano PGH2 in terms of contractile action. The additional contractions seen with high concentration of the 16,16-dimethyl analogue on the bovine iris sphincter could be due to this type of thromboxane activity.

The large variation in the dose-ratio measurements for EP 011 with and without EP 116 and the relatively constant relationship between the PGE2 and the EP 011 dose-response curves in the presence of a high concentration of EP 116 indicate that EP 011 may possess significant PGE2-like activity. A similar argument may be applied to 15-oxo EP 011 but this compound is about 4 times less active than EP 011 in terms of the PGE2-like contractile activity. Recently, Jones (unpublished data) has shown that when 11,9-epoxymethano PGH2 is used as an agonist on the bullock iris sphincter, dose-ratios greater than 100 can only be obtained with high concentrations of EP 092 on those preparations which are relatively insensitive to PGE2. This indicates that high concentrations of 11,9-epoxymethano PGH2 can act on PGE2 receptor sites, too.

It has been shown that ICI 79939 PGF2a and ICI 79939 PGE2 have TxA2-like activity (Jones, Peesapati & Wilson, 1982). On the rabbit aorta equipotent molar ratios are 3.1 for ICI 79939 PGF2a and 4.3 for ICI 79939 PGE2 (11,9-epoxymethano PGH2=1.0). Moreover, on the guinea-pig ileum where PGE2 is the most active substance,

ICI 79939 PGF2a was fairly active (Welburn & Jones, 1978). It is unlikely in the latter study that ICI 79939 PGF2a exerts its contractile action by binding to PGF2a or TxA2 receptors since ICI 81008, a selective PGF2a analogue, and 11,9-epoxymethano PGH2 were very weak on the guinea-pig ileum preparation (Welburn & Jones, 1978; Kennedy, Coleman, Humphey, Levy & Lumley, 1982). In order to study the PGE2-like activity of ICI 79939 compounds on the bullock iris sphincter preparation TxA2 receptor antagonists were used to block the TxA2-sensitive system. In the presence of EP 045 ICI 79939 PGE2 is 6 times more active than PGE2, and in the presence of EP 045 or EP 116 ICI 79939 PGF2a is about half as active as PGE2. PGD2, PGI2 and ICI 81008 are very weak agents on this preparation. These findings indicate that ICI 79939 PGF2a may have potent agonist actions on PGE2-sensitive sites. This idea is supported by Jones's recent work (unpublished data), which shows that EP 092 at a concentration as high as 1.0 μM, produces no or very weak blocking effect on the response to ICI 79939 PGF2a on the bullock iris sphincter.

The low threshold concentration and higher maximum of M/B 28767 imply that M/B 28767 may interact with both PGE2- and TxA2-sensitive sites, even in the presence of a TxA2 receptor blocker. And M/B 28767 may have marked TxA2-like action since its concentration-response curve shifts further to the right as an increasing concentration of EP 045 was added.

Indomethacin produces an inhibitory effect on smooth muscle

contraction induced by varied stimulants (Northover, 1977). It has been reported that contractile responses of smooth muscle to prostaglandins were inhibited more completely by indomethacin than responses to other spasmogens (Sorrentino, Capasso & Dirosa, 1972). The concentrations of indomethacin used by these investigators were in excess of 10 μM. In this study we have shown that indomethacin at 1.0 μM has no or little effect on responses to prostaglandins. At higher concentrations indomethacin inhibited PGE2 action, but did not affect response to 11,9-epoxymethano PGH2.

EP 116 is about 100 times more potent than EP 045 in terms of blocking the response to 11.9-epoxymethano PGH2. The inability of EP 116 to inhibit the spontaneous rise in tension probably indicates that the increase in tension is not due to endogenous synthesis of TxA2 in the bullock iris sphincter. In addition, the tension could not be depressed by atropine or phentolamine. We assume that in addition to TxA2 receptor blocking activity EP 045 may perhaps possess another activity ---- inhibiting endogenous synthesis of PGE2. It is unlikely that EP 045 produced the inhibition in tone by affecting leukotriene synthesis since indomethacin, which has no effect on leukotriene synthesis, suppressed the increased tone.

The slow on-set for certain lipophilic PG analogues at low concentrations may indicate that PG receptors are situated adjacent to a lipophilic minimilieu. The lipophilic agonists will concentrate themselves in this area, then bind to their receptors. The time course of equilibrium between

receptor-bound and free agonists will depend on the concentrating speed of the agonist. Therefore, equilibrium in the minimilieu will be established slowly and this will be reflected in a slow on-set of the contractile effect. It is apparent according to this hypothesis that it is difficult to estimate the true potencies of these lipophilic PG analogues. The potency of the lipophilic agonists may be enhanced due to accumulation of the compounds in the minimilieu.

Horse Iris Sphincter Muscle

The horse iris sphincter muscle possesses at least three PG receptor systems: one is PGF2a-sensitive system mediating contraction only and the other two are PGE2-sensitive systems mediating both contractile and relaxant actions.

The PGF2a receptor site resembles that in the dog and cat iris sphincter. It is characterized by the high agonist activity of ICI 81008, a specific PGF2a mimetic, and the inability of the thromboxane receptor antagonist EP 116 to block the contractile effect.

Evidence for the presence of a PGE2 receptor mediating contraction mainly comes from a study of the activity of 16,16-dimethyl PGE2. This compound is a potent agonist but elicits a maximum response much lower than that obtained with PGF2a. The existence of the PGE2 receptor is further substantiated by the high activity and high maximum of ICI 79939 PGF2a, reflecting the fact that ICI 79939 PGF2a

has both PGF2a and PGE2 activities, as proved in the dog and bullock iris sphincter. Furthermore, ICI 80205, which also has high activity on both PGE2 and PGF2a receptor sites (see bullock, dog and cat iris sphincter) shows the same properties as ICI 79939 PGF2a. Additional support comes from the additive interactions of 16,16-dimethyl PGE2 with PGF2a and of ICI 81008 or PGF2a with PGE2. One can predict that with higher concentrations PGF2a will produce further contractions by activating PGE2 receptors. The observation that ICI 79939 PGF2a has higher activity than ICI 81008 on this preparation is probably due to a summation of the activities of ICI 79939 PGF2a on both PGF2a and PGE2 receptor sites.

It is difficult to study the inhibitory PGE2 system, since a limited relaxant response could be elicited. This PGE2 system appears somewhat different from that in the cat trachea (see cat trachea). In the cat trachea the order of potency for inhibitory activity is PGE2> PGE1>> ZK 36374 > PGI2, PGE2 is about 1000 times more potent than ZK 36374 and PGI2. PGE1 is about half as potent as PGE2. On the horse iris sphincter, PGE1 is more potent than PGE2, and PGI2 and ZK 36374 show relatively high activity. The ostensive relaxant effect produced by low concentrations of PGE2 may however be the algebraic summation of a relaxant and a contractile effect. It is always tempting to think that the "dominated effect" must be small in magnitude. However, it is possible that both contractile and relaxant effects are intense and that the relaxant effect is just powerful enough to give a relaxation. If this is so then the measured

agonist potencies for the relaxant effect may bear little relationship to their true magnitude. Thus, the high activity of PGE1 can be explained by a lower activity of PGE1 on the contractile PGE2 receptor sites than on the inhibitory PGE2 receptor sites in relation to PGE2. The relatively high potency of PGI2 and ZK 36374 may indicate the existence of a PGI2-sensitive system.

The reversal of PGE2 relaxant effect by PGs and carbachol seems to be neither an event which occurs at the receptor levelnor due to potentiation by PGE2 of the contractile actions due to other stimulants, because adrenaline-induced inhibitory effect was also opposed by a PG analogue. Contractile agents were used in the cat trachea and guinea-pig trachea to study PGE2 relaxant action. On the guinea-pig trachea, PGE2 action was not opposed by stimulants (Jones, R.L., Personal Communication), while on the cat trachea high concentration of carbachol opposed the PGE2 relaxant action (Jones, 1970), a case similar to the horse iris sphincter.

It is generally believed that relaxation of smooth muscle and inhibition of platelet aggregation are produced by an increase of cyclic AMP level via activation of plasma membrane receptors. In platelets, agents such as ADP or adrenaline lower the increased cyclic AMP concentration caused by PGE1 (Robison, Arnold & Hartmann, 1969; Ball, Brereton, Fulwood, Ireland & Yates, 1970; Moskowitz, Harwood, Reid & Krishna, 1971; McDonald & Stuart, 1973). One can assume that in the horse iris sphincter carbachol and

other PGs may lower the increase in cyclic AMP concentration induced by PGE2.

The high sensitivity of the preparations to PGF2a tempted us to try 15-oxo EP Ø11 and the like again. Fortunately, tachyphylaxis to the natural agonist PGF2a was not encountered. However, we encountered an additional problem ---- the existence of a PGE2 receptor. As demonstrated before, 15-oxo EP Ø11 at high concentrations activates PGE2 receptors. The contraction elicited by 15-oxo EP Ø11 could be due to activation of both PGF2a and PGE2 receptors. Depending on the sensitivity of the preparation to PGE2, 15-oxo EP Ø11 either shows an "additive" interaction with PGF2a or opposes the action of PGF2a. The desensitization to PGF2a after treatment with 15-oxo EP Ø11 confirms the notion that 15-oxo EP Ø11 acts on the PGF2a receptor sites.

Rabbit Iris Sphincter Muscle

There are two types of PG receptors in the rabbit iris sphincter muscle: PGI2 and PGE2 receptor sites.

In contrast to its inhibitory effect on platelets and many vascular preparations, PGI2 elicits a contractile response on the rabbit iris sphincter. ZK 36374, a stable analogue of PGI2, also produces contraction, and is equipotent with PGI2, which is in agreement with the investigations on bovine coronary artery and human platelets (Schror, Darius, Matzky & Ohlendorf, 1981).

PGEl probably acts as a partial agonist on PGI2 receptor

sites in eliciting the production of cyclic AMP by human platelets (Tateson, Moncada & Vane, 1977). An attempt was made to study whether PGE1 shows partial agonist activity on this preparation. However, the presence of an inhibitory PGE2-sensitive system makes it difficult to analyse the results, since PGE1 is always a potent agonist on PGE2 receptor sites. The low maximum response of PGE1 relative to PGI2 might be due to an inhibitory effect related to an agonist action of PGE1 on PGE2 receptor sites. Nevertheless, that the preparation loses its sensitivity to PGI2 after treatment with PGE1, and PGE1 opposes PGI2 action implies that PGE1 may be a partial agonist on the PGI2 receptor sites mediating contraction, as on the PGI2 receptor sites mediating cyclic AMP production. Obviously, a definitive explanation must await the discovery of specific PGE2 receptor antagonists.

PGE2 receptor sites in this preparation mediate relaxation, as we have mentioned above. That it reduces the tone elicited by both PGI2 and carbachol indicates that its inhibitory effect is not due to acting on PGI2 receptor sites. This is verified by the fact that PGE2-induced inhibitory effect on PGI2 varies with the sensitivity of the preparation to PGE2.

It has been found that PGE2 is about 1000-10000 times less active than PGI2 in inhibiting platelet aggregation induced by collagen or ADP (Andersen, Eggerman, Harker, Wilson & De, 1980). However, PGE2 decreases the effectiveness of PGI2, PGE1 and PGD2 to inhibit platelet aggregation and to

increase cyclic AMP concentration (Andersen et al., 1980; Bonne, Martin, Watada & Regnault, 1981). The latter group also found that PGE2 by itself inhibited platelet aggregation and increased the production of cyclic AMP, and depressed the rise in cyclic AMP induced by adenosine and sodium fluoride. These findings suggest that PGE2 counteracts the antiaggregating action of PGI2 and PGE1 by inhibiting the adenylate cyclase system. However, PGE2 was as potent as PGE1 to displace (3H)-PGE1 from its high affinity specific binding sites in platelet suspension, but did not displace PGD2 from its own receptor (Bonne et al., 1981). It has been suggested that PGE1 and PGI2 share a receptor distinct from that acted on by PGD2 (Whittle, Moncada & Vane, 1978; Miller & Gorman. 1979), and PGI2 is the natural or endogenous ligand for the PGE1 receptor. And it has been shown that a type of PGE2 receptor exists in platelets which potentiates platelet aggregation induced by TxA2 (Weiss, Willis, Kuhn & Brand, 1976). Since PGE1 is more active on PGE2 receptors than on PGI2 receptors and the concentration of (3H)-PGE1 used was 5 nM in the study of Bonne and his colleagues (1981), the labelled ligand probably bound mainly to PGE2 receptors. However, the possibility cannot be entirely precluded that PGE2 is an antagonist or a partial agonist on PGI2 receptor sites. To prove that one needs either a preparation which contains PGI2 receptors only or PGE2 antagonists.

It has been shown that the rabbit iris converts 1-[14C]-arachidonic acid into 6-keto-PGF1a, a PGI2 metabolite, and PGE2 (van Alphen, Dutilh & de Deckere, 1978;

Bhattacherjee, Kulkarni & Eakins, 1979). Since PGs can not be stored, but are synthesized and released when required in response to stimuli, it is reasonable to assume that they should have biological targets in or near the tissue where they are synthesized. The conversion of arachidonic acid into 6-keto PGF1a and PGE2 may support our conclusion that there are PGI2 and PGE2 receptors in the rabbit iris sphincter, unless, of course, these PGs are produced by the iris dilator muscle. Intriguingly, the same investigators have also found that the cat iris is capable of transforming arachidonic acid into 6-keto PGF1a and PGE2 in high proportion. However, PGE2 and PGI2 are very weak agonists on the cat iris sphincter and dilator muscle (see our results; van Alphen & Angel, 1975). According to our hypothesis, PGI2 and PGE2 must have targets in or near those muscles, mediating biochemical or physiological events rather than the mechanical responses, possibly changing membrane permeability to ions or activity of an enzyme, eliciting blood vessel response in a tissue, or modifying neurotransmitter release. Therefore, we have to point out that in this study we define and differentiate PG receptors only in terms of mechanical responses or platelet aggregation.

Section Two

Actions of Prostanoids on the Cat Trachea

Reference citations : pp 355 - 370

INTRODUCTION

The pharmacological effects of prostaglandins on the respiratory tract were first described by Main (1964) and Horton and Main (1965) in several mammalian species. Tracheal or bronchial smooth muscle of several species including man is relaxed in vitro by both PGE1 and PGE2 (Main, 1964; Horton & Main, 1965; Sheard, 1968; Sweatman & Collier, 1968; Mathe, Strandberg & Astrom, 1971).

PGF2a was discovered first in extracts of the lung (Bergstrom, Dressler, Krabigh, Ryhage & Sjovall, 1963). PGF2a contracts tracheal and bronchial smooth muscle including human tissue both in vitro and in vivo (Anggard & Bergstrom, 1963; Sweatman & Collier, 1968; Mathe, Strandberg & Astrom, 1971). Polyphloretin phosphate blocks the bronchoconstrictor action of PGF2a but not the bronchodilator action of PGE2.

TxA2 is the most powerful bronchoconstrictor in the guinea-pig (Svensson, Strandberg, Tuvemo & Hamberg, 1977).

PGI2, which can be formed in lung tissue (Gryglewski, Korbut & Ocetkiewicz, 1978; Omini, Brunelli, Folco, Marini, Pasargiklian & Berti, 1981) shows bronchodilator effects in vivo in dogs and guinea-pigs (Wasserman, Ducharme, Wendling, Griffin & Degraaf, 1980) and is more potent than PGE1 in relaxing human respiratory tract smooth muscle in vitro (Karim, Adaikan & Kottegoda, 1980).

It is of interest that the cat trachea is not contracted by either PGF2a or TxA2. Contractions of the smooth muscle produced by other stimulants are however inhibited by PGs, of which prostaglandins of the E series are the most potent.

A review of the literature indicates that two subtypes of PGE receptor may exist. For example, on the guinea-pig ileum the contractile action of PGE2 on the longitudinal muscle is blocked by both the benzoxazepine SC-19220 and polyphloretin phosphate (PPP) whereas its relaxant action on the circular muscle is unaffected by those two agents (Bennett & Posner, 1971). In addition. the contractile action of PGE2 on the rat stomach fundus is blocked by SC-19220 but contraction of the chick ileum is not (Coleman, Kennedy, Levy & Penning, 1980).

We have found that 16,16-dimethyl PGE2 and ICI 80205 are potent agonists, and ZK 36374 is a partial agonist on PGE2 receptor sites mediating an excitatory response in the bullock iris sphincter. Attracted by the unique properties of cat trachea, i.e. PGs cause an inhibitory response only, we have examined the PGE2 analogues and ZK 36374 on this preparation and attempted to provide further evidence for the existence of receptor subtypes for PGE2.

METHODS

The cat trachea was dissected out under pentobarbitone anaesthesia, and cut transversely between the segments of cartilage. Two of these rings were tied together to form a chain and then the cartilage side was cut off. The preparations were suspended in 10 ml organ baths containing Krebs solution (NaCl 6.9, KCl 0.35, $MgSO_4 \cdot 7H_2O$ 0.29, KH_2PO_4 0.16. glucose 2.0, $NaHCO_3$ 2.1, $CaCl_2$ 0.28 g/l), gassed with 95% O_2 and 5% CO_2 , and kept at 37°C.

Tension changes were measured isometrically with a Grass force displacement transducer (FT 03C) and recorded on a Grass Polygraph (Model 7C). Each preparation was allowed 1 h to stabilize.

RESULTS

The cat tracheal muscle contracts to carbachol. (an acetylcholine analogue) and 5-hydroxytryptamine(5-HT). The concentrations which produced a response 50% of their own maximum were in a range of 0.1-0.7 μM, mean=0.32 ± s.e.0.042 μM (n=21). for carbachol and 2.0-5.0 μM for 5-HT. PGE2. PGF2a, PGD2, 11.9-epoxymethano PGH2, PGI2 and ZK 36374 produced neither contractile nor relaxant effects on this preparation when it was in the resting state. As the cat trachea preparation possesses negligible tone in vitro, it was contracted with carbachol at concentrations between 0.05 and 0.2 μM to give a response about 60% of its own maximum so that the inhibitory effect of a prostanoid could be investigated. In a few preparations 5-HT (2.0 μM) was used for this purpose. Prostanoids were added to the organ bath after the dose of carbachol or 5-HT had produced a sustained contraction. The response to the fixed concentration of carbachol was highly reproducible. Doses of prostanoids were added cumulatively. Figure T.1 illustrates a typical trace of responses of the cat tracheal muscle preparation to cumulative doses of PGE2. Responses to PGE2 reached their nadir in 2-4 min. In most cases responses to PGE2 were sustained for at least 2 min or more, but in a few cases responses showed a tendency to return to the tone level provided by carbachol. After wash-out of the organ bath, the tension of the muscle returned to the resting level. The interval between two sequences was about one hour. The

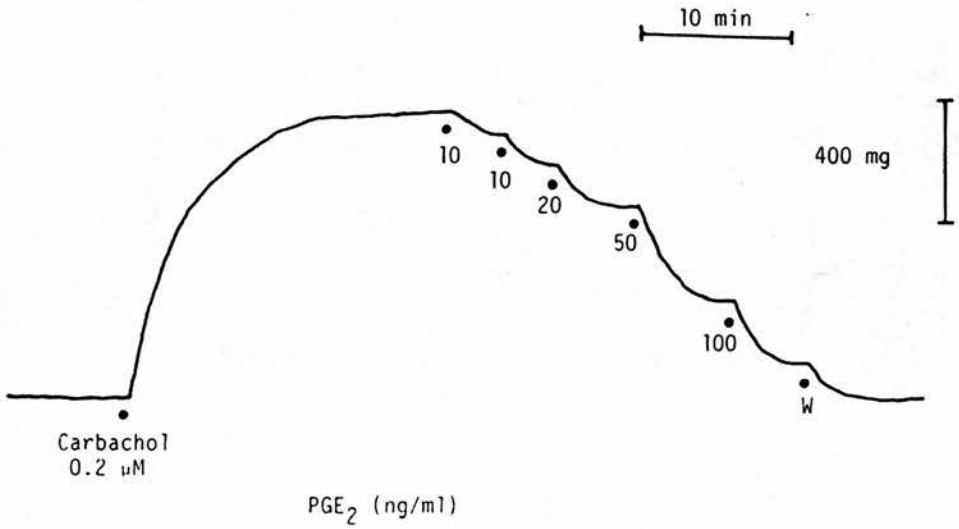


Figure T.1 Cat tracheal preparation: responses to cumulative dose of PGE_2 The organ-bath contained Krebs' solution. Tone was provided with 0.2 μM carbachol. W=wash.

response was taken as the reduction in the carbachol-produced tension. Log concentration-response curves were then plotted.

PGE2 Analogues

PGE2 is a potent relaxant agent and was used as the standard agonist. In most cases, 15 out of 19, threshold responses (tension change of about 100mg) were seen with concentrations of 3-10 ng/ml and 50% maximum responses with 18-60 ng/ml. In one preparation which was very sensitive to PGE2 the EC50 was 0.28 ng/ml and in three preparation EC50 values were 115,130 and 170 ng/ml. Representative log concentration-effect curves for PGE2, PGE1, 16,16-dimethyl PGE2 and ICI 80205 are shown in Figure T.2a and T.2b. Log concentration-effect curves for these compounds were parallel to that of PGE2. Equipotent molar ratios were obtained from the molar concentration of PGE2 giving a response 50% of its own maximum and the molar concentration of the test compound producing a commensurate response (Table T.1). On ordinary preparations PGI2 and ZK 36374 at 1 µg/ml produced no effect and ZK 36374 did not oppose the inhibitory effect of PGE2 (Figure T.3). On the one highly PGE2-sensitive preparation (the threshold concentration was 0.2 ng/ml) ZK 36374 and PGI2 showed relaxant effects (Figure T.4). PGE2 was about a thousand times more active than ZK 36374 and PGI2.

PGF2a Analogues

PGF2a at a concentration of 100 ng/ml relaxed the

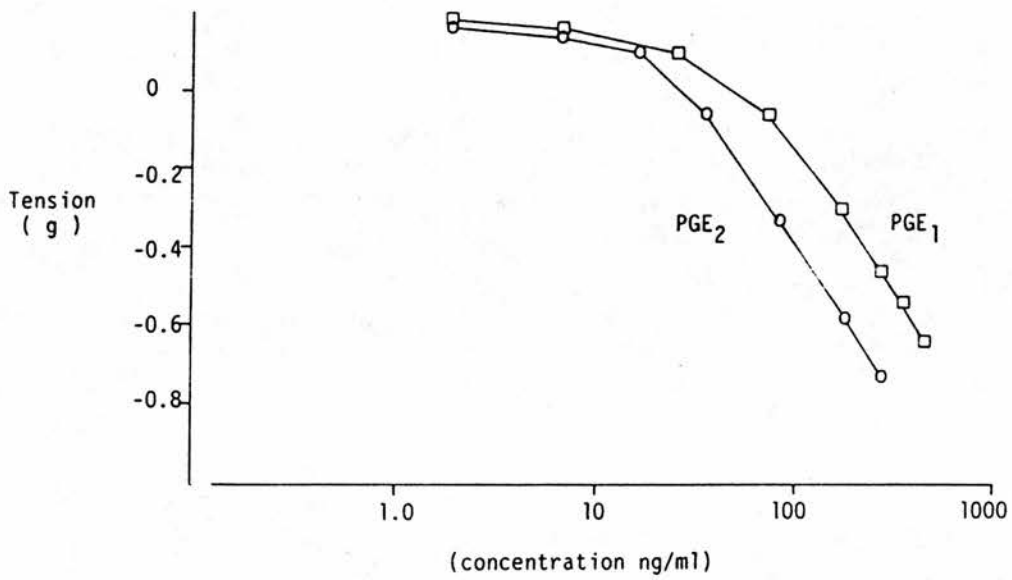


Figure T.2a Cat tracheal preparation: log concentration-response curves for PGE_2 and PGE_1. Tone was provided with 0.2 μM carbachol.

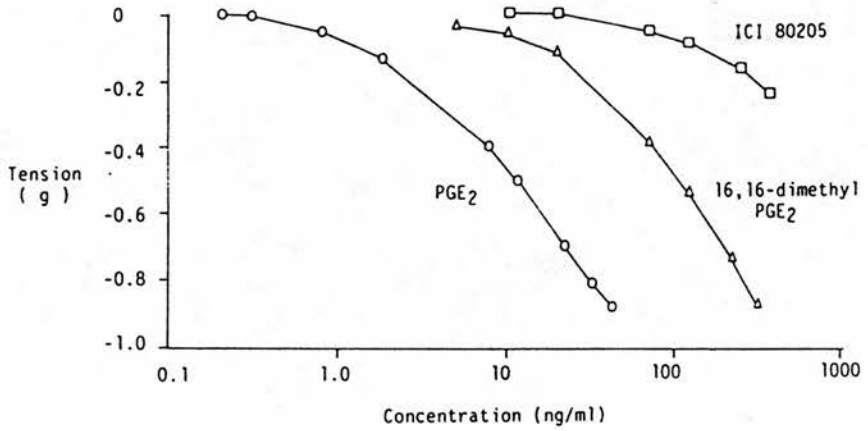


Figure T.2b Cat tracheal preparation: log concentration-response curves for PGE_2, 16,16-dimethyl PGE_2 and ICI 80205. Tone was provided with 0.3 μM carbachol.

Table T.1 Potencies of prostanoids on the cat tracheal muscle preparation

Compound	Equipotent molar ratio (PGE_2=1.0) Individual value	mean s.e.	n
PGE_1	1.3 2.3 2.6 3.0	2.3±0.36	4
16,16-dimethyl PGE_2	7.4 8.3 9.3 14	9.8±0.73	4
ICI 80205	69 75 90	78±6.2	3

Individual values are the result of a comparison with PGE_2 on a single preparation. With ICI 80205 the full concentration-response relationship was not established, and the corresponding equipotent molar ratios relate to response at the 25% maximum response level.

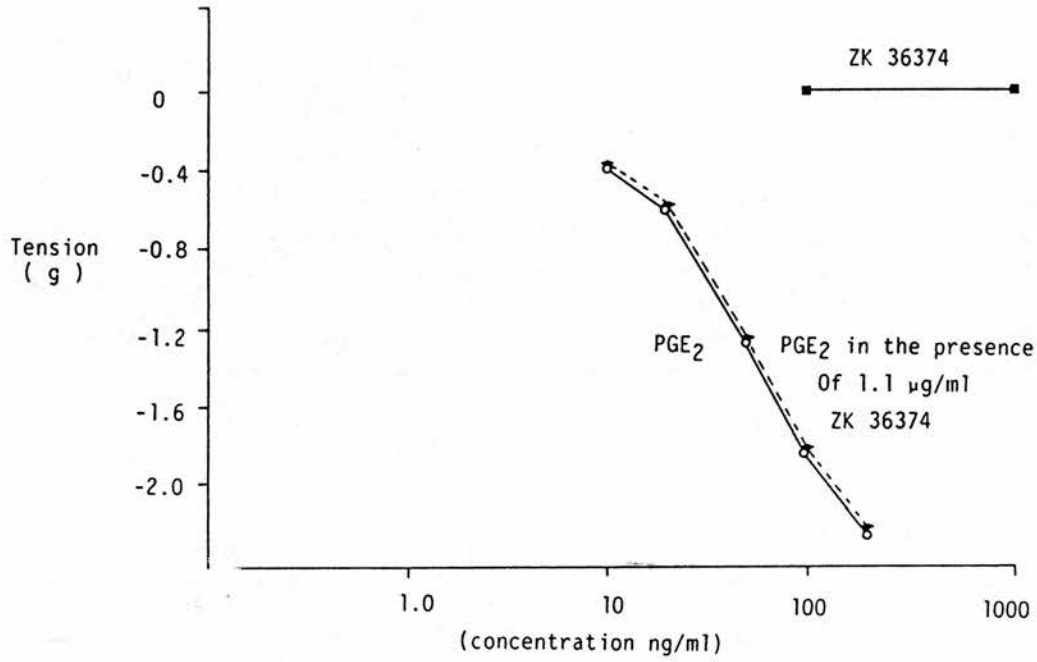


Figure T.3 Cat tracheal preparation: log concentration-response curves for PGE_2 alone and PGE_2 in the presence of 1.1 µg/ml ZK 36374. Tone was provided with 1 µM carbachol.

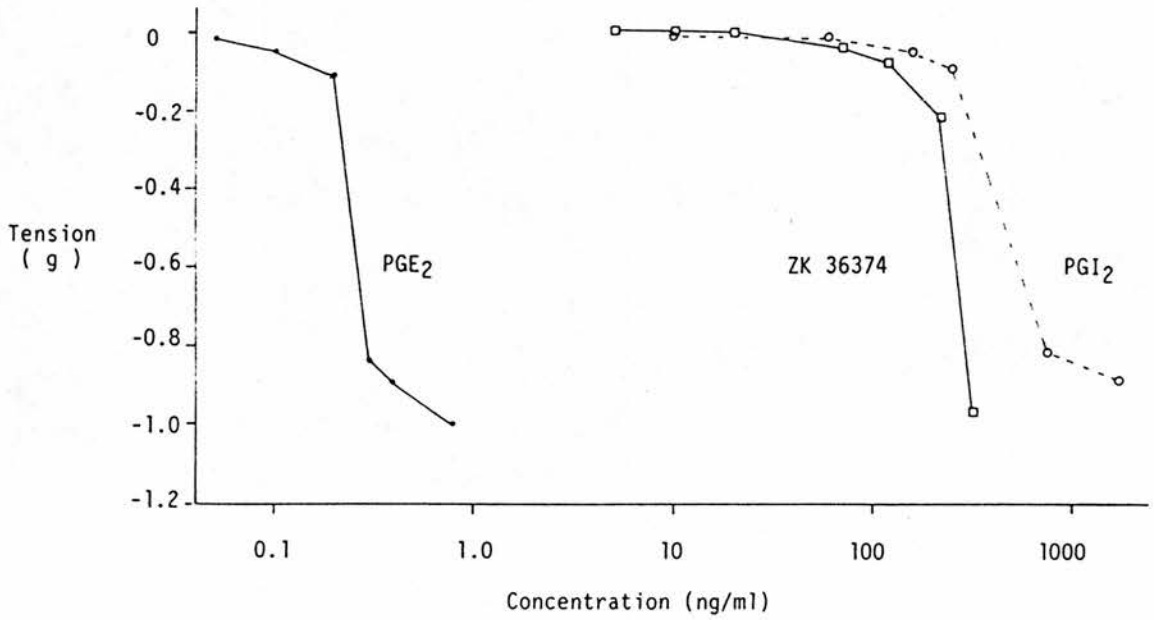


Figure T.4 The highly PGE_2-sensitive cat tracheal preparation: log concentration-response curves for PGE_2, ZK 36374 and PGI_2. Tone was provided with 0.1 μM carbachol.

preparation, but the response faded rapidly. Subsequent addition of PGF2a failed to produce a relaxation (Figure T.5). Propranolol at 1 μM did not oppose the relaxant effect of PGF2a, but antagonized the action of adrenaline which showed relaxant activity on the cat tracheal muscle preparation. PGF2a also caused relaxation when the preparation was contracted with 5-HT and again tachyphylaxis followed the relaxation. The sensitivity of the preparation to PGE2 was not altered by the desensitization to PGF2a (see Table T.2).

Some other PGF2a analogues were also studied. It was found that ICI 81008, ICI 79939, $PGF_{2\alpha}$ and 13,14-didehydro PGF2a (at 100 ng/ml) behaved in the same way as PGF2a, producing a relaxant effect followed by tachyphylaxis. There was cross-desensitization between ICI 81008 and PGF2a (Figure T.6).

Effect of Other Drugs

PGD2, 11.9-epoxymethano PGH2, EP 011 and 15-oxo EP 011 at 100 ng/ml showed no relaxant effect on most of the cat tracheal muscle preparations. On one preparation 15-oxo EP 011 at 500 ng/ml gave a small relaxant effect. Although tachyphylaxis to 15-oxo EP 011 occurred, it seemed not to affect the response to PGF2a and PGE2.

The effects of drugs on the relaxant response to PGE2 are summarized in Table T.2. EP 045, 15-oxo EP 011 and ZK 36374 did not show either contractile or relaxant activities on the preparations where they were tested. Verapamil depressed

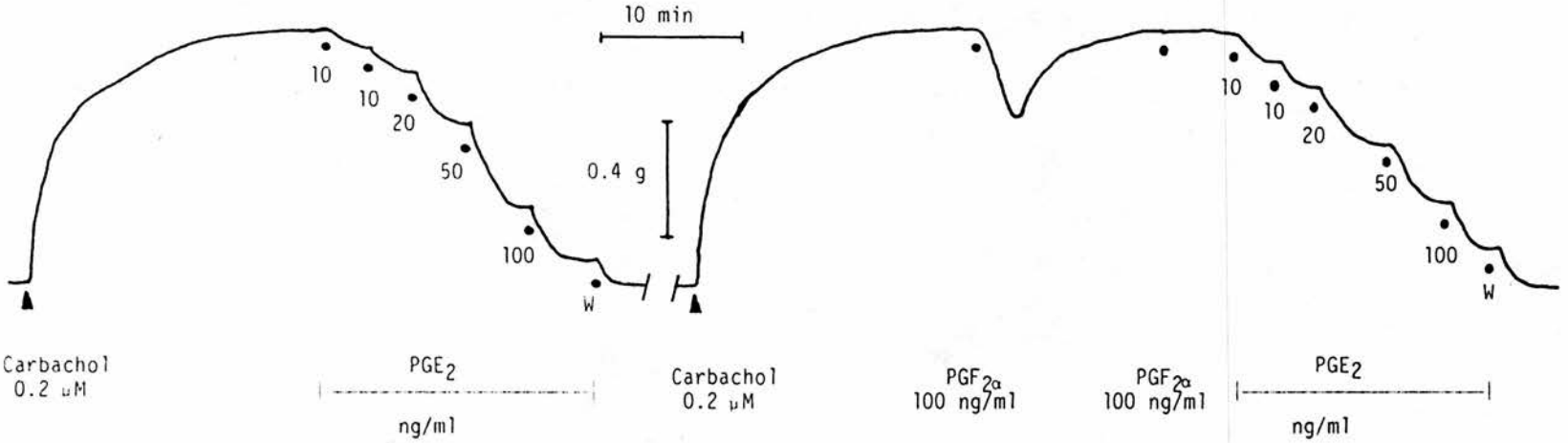


Figure T.5 Cat tracheal preparation: desensitization to $PGF_{2\alpha}$. Two consecutive sequences were carried out in a single preparation. The interval was about an hour. In the first sequence, PGE_2 alone was tested; in the second sequence, $PGF_{2\alpha}$ 100 ng/ml was added into the organ-bath. After tachyphylaxis occurred, a second dose of $PGF_{2\alpha}$ (100 ng/ml) was added into the organ-bath. It produced no effect. Then a repeated concentration-response relationship for PGE_2 was set up in the presence of 200 ng/ml $PGF_{2\alpha}$. The preparation showed no change in the sensitivity to PGE_2. Tone was provided with 0.2 μM carbachol. W = wash.

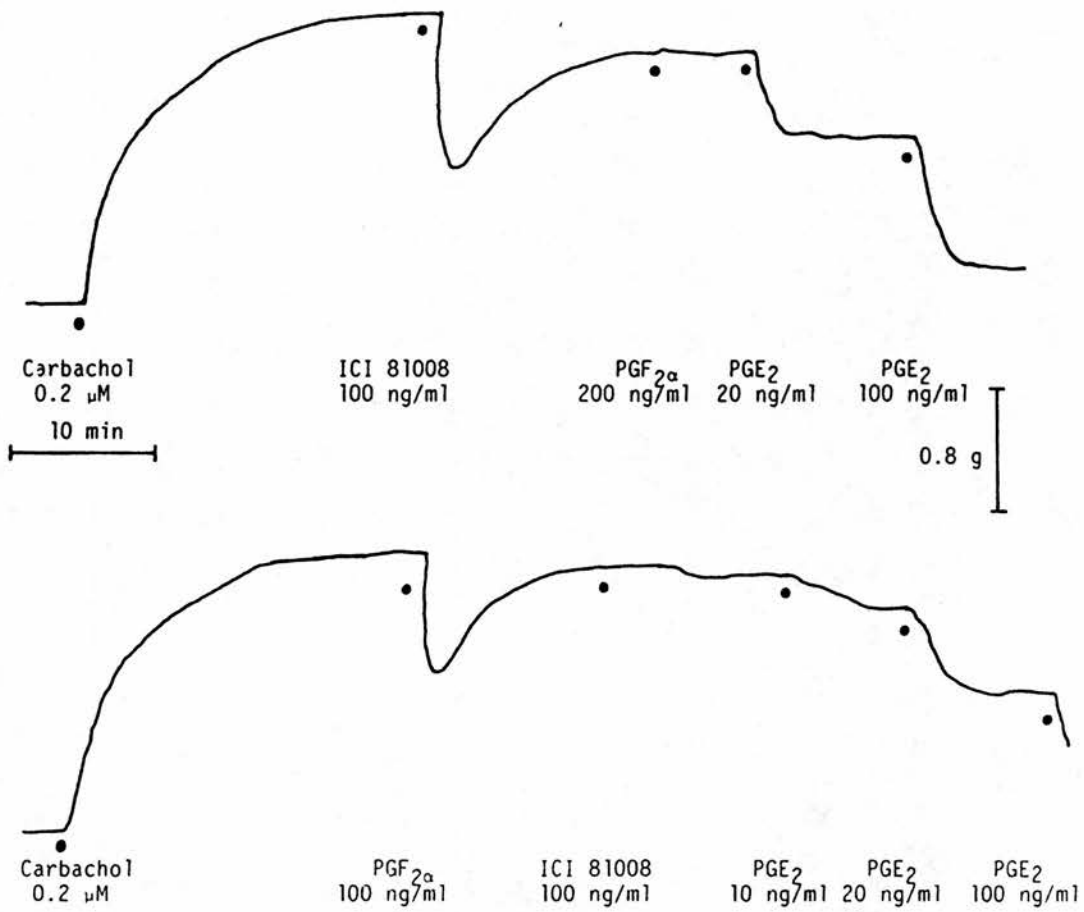


Figure T.6 Cat tracheal preparation: cross-desensitization to $PGF_{2\alpha}$ and ICI 81008. The experiments were conducted on two separate preparations. Having been challenged with $PGF_{2\alpha}$ and ICI 81008, the preparations were still sensitive to PGE_2. The organ-bath contained Krebs solution. Tone was provided with carbachol 0.2 μM.

Table T.2 Effects of other drugs on relaxant responses to PGE_2 or PGE_1 on the cat tracheal muscle preparations.

Treatment		Dose-ratios for PGE_2 or PGE_1
Compound	Dose (μg/ml)	(PGE_2 or PGE_1=1.0)
PGE_2 as agonist		
control (second sequence)		0.71 1.0
EP 045	1	0.68 0.75 0.86 0.89 (0.80±0.049, n=4)
verapamil	10 μM	1.1 2.1 3.7
$PGF_{2\alpha}$	0.2	1.0 2.4
	1.1	1.4
15-oxo EP 011	0.5	0.67
EP 011	1.2	1.1
ZK 36374	1.1	1.0 1.3
	1.6	0.84
	2.0	1.0
ICI 81008 + $PGF_{2\alpha}$	0.1 0.2	0.67
$PGF_{2\alpha}$ + ICI 81008	0.1 0.1	0.67
PGE_1 as agonist		
$PGF_{2\alpha}$	0.5	0.80

Values in the parenthesis indicate mean±s.e..

the contractile response to carbachol, its pA2 value was about 6.Ø. In order to study the relaxant effect of PGE2 in the presence of verapamil, the concentration of carbachol was increased to give a similar tone level to that produced by carbachol alone. Verapamil slightly shifted the PGE2 dose-response curve to the right. EP Ø45 neither blocked the relaxant effect of PGE2 nor the contractile effect of carbachol, it raised slightly the tone produced by carbachol.

DISCUSSION

The results suggest that the cat tracheal muscle has two types of prostanoid receptorswhich mediate relaxation. One is sensitive to PGE2 analogues and is not readily desensitized. The other type responds to PGF2a analogues and is readily desensitized.

On the PGE2 receptor site, PGE2 is the most potent agonist among the naturally-occurring prostanoids as shown by Horton and Main (1965) and Apperley. Coleman. Kennedy & Levy (1979). The order of potency for PGE2 and PGI2 analogues is PGE2> PGEl> 16.16-dimethyl PGE2> ICI 80205>> ZK 36374> PGI2. and ZK 36374 does not oppose the relaxant effect of PGE2. In contrast, ICI 80205 and 16,16-dimethyl PGE2 are more potent than PGE2 on the bullock iris sphincter, rat stomach fundus and rat anococcygeus muscle, and ZK 36374 is a partial agonist on these preparations (see bullock iris sphincter, rat stomach fundus and rat anococccygeus muscle). These results indicate that the PGE2 receptor mediating the relaxant effect on the cat tracheal muscle is different from that mediating the contractile effect on the other preparations mentioned above. This is substantiated by the finding that SC-19220 caused concentration-related parallel shifts to the right of dose-response curves for PGE2 on PGE-sensitive preparations such as the guinea-pig ileum and guinea-pig and dog fundus, but had no effect on PGE2-induced relaxation of cat trachea (Kennedy, Coleman, Humphrey, Levy & Lumley,

1982).

We have found (data not shown) a similar PGE2 receptor system in the dog hind limb where PGE2 is the most potent prostanoid, PGE1, 16,16-dimethyl PGE2 and ICI 80205 are weaker than PGE2. However, PGI2 and ZK 36374 show higher activity in the dog hind limb than in cat trachea preparation relative to PGE2. This awaits more work to exclude a PGI2 receptor site in the dog hind limb.

The other type of prostanoid receptor shows a distinctive property ---- tachyphylaxis to PGF2a analogues. It seems not to be due to inactivation of PGF2a on the PGF2a-desensitized preparation since successive addition of PGF2a produced no effect and ICI 81008 and ICI 79939, stable PGF2a analogues induced tachyphylaxis, too. It is unlikely that PGF2a analogues act on PGE2 receptor sites to produce such an effect since the PGF2a analogue—desensitized preparations maintained the same sensitivity to PGE2 as before they were treated with PGF2a analogues.

Tachyphylaxis to prostanoids has been observed previously both in vitro and in vivo. Marked tachyphylaxis on the rat uterus preparation was demonstrated with several prostaglandins (Adamson, Eliasson & Wiklund. 1967; Eliasson, Brzdekiewicz & Wiklund, 1969). In experiments on anaesthetised chicks PGF2a injected intravenously increases gastrocnemius muscle tension due to an action of PGF2a on neurones within the chick spinal cord (Horton & Main, 1967) and tachyphylaxis to successive doses of PGF2a often develops if the interval between doses is short.

Tachyphylaxis to the action of PGF2a has also been observed on application to single neurones in the brain stem (Avanzino, Bradley & Wolstencroft, 1966). In dogs exhibiting marked sinus arrhythmia injection of PGE2 through a common carotid artery abolishes or markedly reduces the arrhythmia and tachyphylaxis occurred after 2 or 3 injections repeated at 20 min intervals (McQueen & Ungar, 1969). In human isolated bronchial smooth muscle tachyphylaxis to SRS-A and PGF2a could be produced and there was no cross tachyphylaxis between these substances (Sweatman & Collier, 1968). Although the significance of tachyphylaxis to prostaglandins is not clear, the effect can be a useful tool for defining and differentiating receptors. Agonist-specific desensitisation of receptor-adenylate cyclase systems has been used to probe prostanoid receptors in several tissues (Remold-O Donnell, 1974; Newcombe, Ciosek, Ishikawa & Fahey, 1975; Su, Cubeddu & Perkins, 1976; Lefkowitz, Millikin, Wood, Gore & Mukherjee, 1977). Cooper and Schafer, Puchalsky and Handin (1979) reported that pre-incubation of human platelets with PGD2 (10 μM) for 2 h followed by washing desensitized platelet adenylate cyclase to PGD2 more than to PGE1. In addition, Miller and Gorman (1979) showed that prior exposure of human platelets to PGE1 (0.28 μM), PGE2 (2.8 μM) or PGI2 (0.28 μM) for 5 min rendered platelets desensitised to a subsequent challenge by these prostaglandins but not to PGD2. The corollary was also true: preincubation with PGD2 (0.28 μM) desensitised platelets to a subsequent challenge with PGD2, but responses to PGE1, PGE2 or PGI2 were unaltered.

PGF2a does not exert its inhibitory effect by releasing neurotransmitters from the adrenergic nerve endings as propranolol failed to antagonize PGF2a action. And in view of the weak activities of PGD2, PGI2 and 11,9-epoxymethano PGH2 it is reasonable to assume the there is a PGF2a type of receptor mediating relaxation in the cat tracheal muscle. Ligand binding measurements would be required to make further differentiation between PGE2 and PGF2a receptors in this preparation.

The relaxation and tachyphylaxis produced by PGF2a analogues were not specific for carbachol-contracted preparations; similar activity was seen on 5-HT-contracted preparations. Thus, the inhibitory effect of PGF2a is probably not specifically associated with either acetylcholine or 5-HT receptors.

In this study a contractile agent concentration which produced a response about 60% of its own maximum was chosen. Increasing the contractile agent concentration results in reduced relaxant responses to prostanoids, as demonstrated by Jones (1970) (Figure T.7). This was further proved by two separate groups of experiments. In one group of experiments where low concentrations of acetylcholine (about 0.1 μM) were used, PGE1 showed high activity (threshold concentration <2 ng/ml) (Horton & Main. 1965); in the other group of experiments where a high concentration of acetylcholine (about 55 μM) was used, the EC50 value for PGE2 was about 350 ng/ml (Kennedy, Coleman, Humphrey, Levy & Lumley, 1982). And acetylcholine concentrations affect

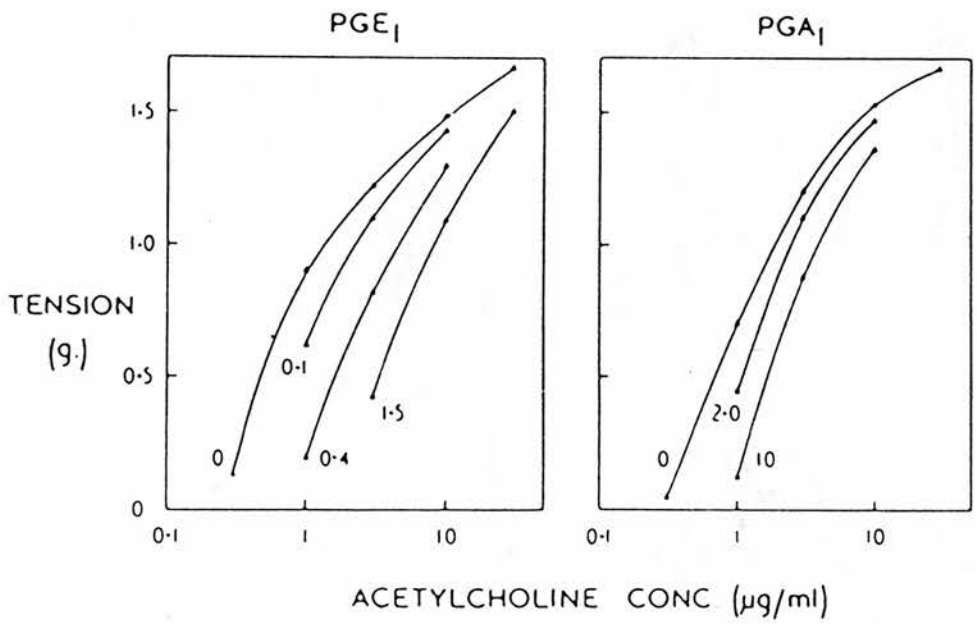


Figure T.7 Cumulative dose response curves to acetylcholine chloride on the cat tracheal chain preparation in the presence of PGE_1 and PGA_1. Figures adjacent to the curves refer to PG concentrations in µg/ml. Ordinate: tension in g. Abscissa: acetylcholine concentration in µg/ml (logarithmic scale). (Data from Jones R.L., 1970, Ph.D. Thesis).

responses to PGF2a as well. In the former group, the activity of PGF2a was about one-thirtieth of the PGEs and response to PGF2a took a "hook" shape: a rise in tone followed a relaxant response (Figure T.8), but in the latter group PGF2a was about 100 times less active than PGE2. The use of an appropriate concentration of the contractile agents. enabled us to show in this study the high activity of PGE2 and the tachyphylaxis to PGF2a.

In anesthetized, vagotomized and mechanically ventilated cats bronchoconstricted by 5-HT, PGE2 produced a greater reduction in central airway constriction than PGI2, and 6-keto PGE1, a PGI2 metabolite, was also more potent than PGI2 (Spannhake, Levin, Hyman & Kadowitz, 1981). According to the results from this study it is possible that 6-keto PGE1 acts on PGE2 receptor site to produce the bronchodilator effect.

That verapamil, a calcium channel blocker, failed to affect relaxant responses to PGE2 probably indicates that this inhibitory effect of PGE2 does not rely on extracellular calcium.

EP 045 does not influence the responses to PGE2 and carbachol. This further shows the selectivity of EP 045. The rise in tone caused by EP 045 may be due to its inhibition of the biosynthesis of endogenous PGE2 in the trachea.

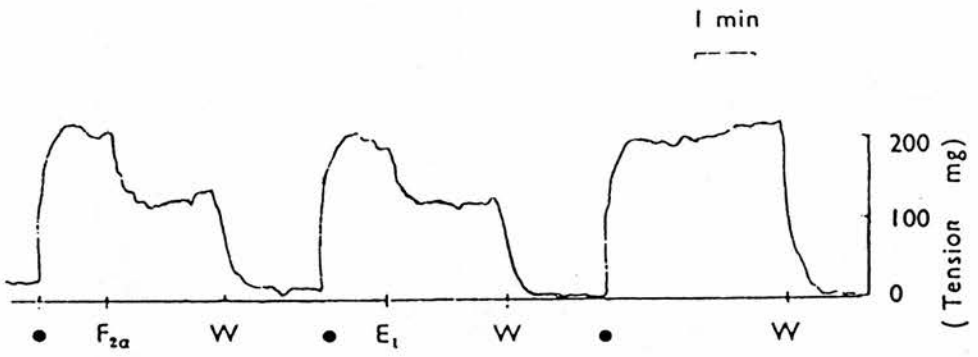


Figure T.8 Isometric responses of cat isolated trachea, suspended in 4 ml organ-bath containing Krebs-Henseleit solution. At the dots acetylcholine 12.5 ng/ml was added. F_2 = $PGF_{2\alpha}$, 0.75 µg/ml; E_1 = PGE_1, 1.9 ng/ml; W = wash. The "hook" shape can be seen in the response to $PGF_{2\alpha}$. (Data from Horton E.W. & Main I.H.M., 1965, Br.J.Pharmac. 24, 470-476)

Section Three

Actions of Prostanoids on the Rat Gastric Fundus and Colon, and Guinea-Pig Ileum and Colon

Reference citations : pp 355 - 370

INTRODUCTION

PGs occur in a wide variety of gastrointestinal tissues. Many studies on gastrointestinal motility have dealt mainly with prostaglandins of the E and F series which until recently were thought to be the major PGs formed by many tissues. In general, PGE2 relaxes circular but contracts longitudinal smooth muscle whereas PGF2a contracts both types. PGD2 produces contractile effect on the longitudinal muscle of rat and human stomach, rabbit jejunum and guinea-pig intestine (Horton & Jones, 1974; Hamberg, Hedqvist, Strandberg, Svensson & Samuelsson, 1975; Bennett, Hensby, Sanger & Stanford, 1981; Bennett & Sanger, 1980), and on the circular muscle of guinea-pig colon (Bennett, Pratt & Sanger, 1980). On both longitudinal and circular muscle preparations from human stomach 11.9-epoxymethano PGH2 is the most potent excitatory prostanoid. This contrasts with gastro-intestinal preparations from laboratory animals where PGE2 is thought to be the most potent prostanoid, although PGF2a or TxA2 may have considerable potency in some tissues. For instance, TxA2 contracts the rat gastric fundus, and is about 10 times less potent than PGE2 (Bunting, Moncada & Vane, 1976). In guinea-pig ileum, 11,9-epoxymethano PGH2 is about 100 times less active than PGE2 (Chijimats et al., 1977; Bennett et al., 1978). PGI2 seems less potent than other prostanoids in most of these preparations.

Recently, several drugs have been examined as antagonists of

various prostanoids in the gut, partly to characterise the types of prostanoid receptors present (Bennett, Jarosik, Sanger & Wilson, 1980; Bennett, Pratt & Sanger, 1980; Sanger & Bennett, 1980; Sanger & Bennett, 1980a). The antagonists are the benzoxazepine derivative SC-19220, the beta-adrenoceptor stimulant trimethoquinol and the PG synthesis inhibitors sodium meclofenamate and indomethacin. On the longitudinal muscle of rat gastric fundus SC-19220, trimethoquinol and sodium meclofenamate show differing blocking activity and this may indicate the presence of different types of prostanoid receptor. PGE2 probably acts on receptors different from those activated by 11,9-epoxymethano PGH2, since SC-19220, meclofenamate or trimethoquinol antagonized these compounds to different extents, and in particular, none of the drugs antagonised contractions to both PGE2 and 11,9-epoxymethano PGH2. In addition trimethoquinol reduced contractions to PGE1 but not to PGE2; thus the authors suggested that there are separate receptors for PGE1 and PGE2 (Bennett, Jarosik, Sanger & Wilson, 1980).

It has been proposed that in the longitudinal muscle of guinea-pig ileum, PGE1, PGE2 and PGF2a may activate similar receptors (Bennett & Posner. 1971; Illes & Knoll, 1975). Contractions to PGD2, PGE2 or PGF2a were antagonised with low concentrations of meclofenamate which had little effect on contractions to acetylcholine on this preparation (Bennett, Pratt & Sanger, 1980). Recently. prostanoid receptors in the longitudinal muscle of guinea-pig ileum have been characterised by determining the order of agonist

potency and their sensitivity to antagonism by SC-19220. The order of potency for prostanoid-induced contraction is PGE2> PGE1> PGF2a> 11,9-epoxymethano PGH2> ICI 81008 (Coleman, Humphrey, Kennedy, Levy & Lumley, 1980). SC-19220 antagonised contraction of guinea-pig ileum to PGE2 or PGF2a without greatly affecting that to acetylcholine or histamine; the pA2 values for PGE2 or PGF2a antagonism were similar (5.4 for PGE2 and 5.3 for PGF2a) (Coleman, Kennedy & Levy, 1980), supporting the results of Bennett and Posner (1971) and suggesting PGE2 and PGF2a share the same receptor. Since in the longitudinal muscle of guinea-pig ileum receptors for PGE1 and PGE2 seem similar (Illes & Knoll, 1975), it is probable that PGE1, PGE2 and PGF2a activate the same receptor.

Since we had available several TxA2 receptor antagonists, a specific PGF2a agonist (ICI 81008) and a possible partial agonist on the PGE2 receptor (ZK 36374), we chose the rat gastric fundus and guinea-pig ileum, which are most sensitive to PGE2 analogues, in order to differentiate further the PG receptors on these preparations and to test whether ZK 36374 is a partial agonist on other PGE-sensitive systems. At the same time certain other preparations from these two species were also studied.

METHODS

Gastric fundi and colons were removed from male Wistar rats, and ilea and colons from male guinea-pigs immediately after they had been stunned and bled.

Two strips of gastric fundus, about 1.5 cm long and 2 mm wide, were cleaved parallel to the longitudinal muscle fibres, one from each side of the greater curvature. Each preparation was mounted under 1.0 g tension in a 10 ml organ bath containing Krebs solution (NaCl 6.9, KCl 0.35, $MgSO_4 \cdot 7H_2O$ 0.29, KH_2PO_4 0.16, glucose 2.0, $NaHCO_3$ 2.1, $CaCl_2$ 0.28 g/l). gassed with 95% O_2 and 5% CO_2. and kept at 37°C. The bathing solution for the rat gastric fundus contained indomethacin 1.0 μM. Responses were measured isotonically with a Washington isotonic lever transducer (Type T.II), and recorded on a Grass Polygraph (Model 7C).

The guinea-pig ileum and colon and the rat colon, each about 2 cm long, were mounted under 0.5 g tension in Tyrode's solution (NaCl 8.0, KCl 0.2, $MgSO_4 \cdot 7H_2O$ 0.26, $NaH_2PO_4 \cdot 2H_2O$ 0.07. glucose 1.0, $NaHCO_3$ 1.0, $CaCl_2$ 0.2 g/l), aerated with air, and kept at 37°C. The bathing solution for the guinea-pig ileum contained atropine sulphate (0.02 μM). The rest of procedure was the same as for rat fundi.

RESULTS

Rat Gastric Fundus

All the prostanoids tested produced concentration-dependent contraction of the longitudinal smooth muscle of rat gastric fundus. PGE2 is the most potent prostanoid. A typical trace of responses of the rat gastric fundus preparation to cumulative doses of PGE2 is shown in Figure G.1. Responses to naturally-occurring prostanoids peaked at 2-4 min and remained steady for at least 20 min. A full dose-response relationship was established in 30-40 min; following wash-out, the response returned to the resting level in about half an hour. The interval between two sequences was about 1 hour. ICI 81008, ICI 79939 PGF2a, 15-oxo EP 011 and EP 011 each displayed a relatively slow on-set of action, the contraction reaching a stable level in 5-10 min. ICI 80205 and 16,16-dimethyl PGE2 were the slowest in on-set. It took 20-30 min for these compounds to produce a stable submaximal response. Therefore, the naturally-occurring prostanoids were always tested first and each comparison of potency was made on a single preparation.

PGE2 Analogues

For the standard agonist PGE2. a mean threshold concentration (tension change of about 5% maximum response) of 0.16 ng/ml (range 0.02-0.25 ng/ml, n=49) was calculated. A 50% maximum response was shown with a mean concentration

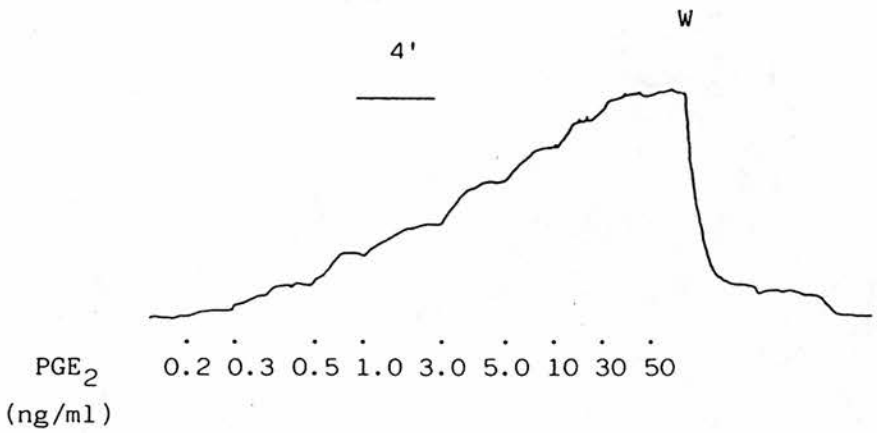


Figure G.1 Rat gastric fundus preparation: responses to cumulative doses of PGE_2. The bathing solution contained indomethacin 10^{-6} M. Changes in response were recorded isotonically. W = wash.

of 1.7 ng/ml (range 0.3-8.8, n=49). The PGE2 analogues examined were PGE2. 16,16-dimethyl PGE2 and ICI 80205. The relative potenciesof these compounds are shown in Table G.1. Equipotent molar ratios were measured using the EC50 value of PGE2 and the molar concentration of the tested compound giving an equivalent response. ICI 80205 is the most potent contractile agent tested on the rat gastric fundus preparation.

In view of the fact that ICI 80205 and 16,16-dimethyl PGE2 have TxA2-like activity and EP 045, a TxA2 receptor antagonist, blocks the action of 11,9-epoxymethano PGH2 on this preparation, a group of experiments were designed to measure the potency of the PGE2 analogues both in the presence and absence of EP 045. The results are summarized in Table G.2. In control experiments (without EP 045) the usual procedure was carried out, but in EP 045-treated preparations 1 μg/ml EP 045 was added 10 min before the start of first agonist sequence (PGE2) and the same dose of EP 045 was added again 10 min before the start of the second agonist sequence (ICI 80205 or 16,16-dimethyl PGE2). Since ICI 80205 and 16,16-dimethyl PGE2 were slow in on-set and off-set, only two concentration-response relationships were established on one single preparation. In both control and EP 045 treated preparation PGE2 was taken as a standard.

Considering the possibility that the sensitivity of the preparation to PGE2 might be changed with time, a second sequence of PGE2 was run in both control (without EP 045) and EP 045-treated preparations. Table G.2 shows that there

Table G.1 Activities of PGE_2 analogues on the isolated rat gastric fundus

Compound	Equipotent molar ratio (PGE_2=1.0) individual value	mean±s.e.	n
ICI 80205	0.015 0.042 0.047 0.059	0.041±0.0093	4
16,16-dimethyl PGE_2	0.14 0.17 0.35 0.36 0.42	0.29±0.056	5
PGE_1	3.0 4.3 5.0 6.5	4.7±0.073	4

Individual values are the result of a comparison with PGE_2 on a single preparation.

Table G.2 Potencies of PGE_2 analogues on the isolated rat gastric fundus in the presence or absence of EP 045

Compound	Equipotent molar ratio (PGE_2=1.0) without EP 045	with EP 045 (1 µg/ml)
PGE_2 (second sequence)	1.1,1.1,1.1,1.6 (1.2±0.13,n=4)	0.93,1.0,1.4,1.5 (1.2±0.14,n=4)
16,16-dimethyl PGE_2	0.14,0.17,0.35,0.36 (0.26±0.058,n=4)	0.19,0.25,0.29,0.38 (0.28±0.040,n=4)
ICI 80205	0.042,0.047,0.059 (0.049±0.0050,n=3)	0.053,0.060,0.074 (0.062±0.0062,n=3)

For legend see text.

was little change in sensitivity to PGE2 in both cases, and that EP 045 at 1 µg/ml had little effect on responses to ICI 80205 and 16,16-dimethyl PGE2.

Figure G.2a and 2b show representative log concentration-response curves of PGE2 analogues. 16,16-dimethyl PGE2 reached the maximum similar to that of PGE2 both in the presence and absence of EP 045. The log concentration-response curve for ICI 80205 over the concentration range of 0.02-0.5 ng/ml was approximately parallel to that of PGE2. At higher concentrations of ICI 80205 further contractions could be elicited which reached a higher maximum (11 ± s.e.2.5% n=4 for control preparations and 11 ± s.e.2.3% n=4 for EP 045-treated preparations) than that produced by PGE2.

PGI2 Analogues

PGI2 and ZK 36374 was also tested in the rat gastric fundus preparation. PGE2 was used as the reference compound. The sequence was to establish PGE2 concentration-response relationship first, then those of PGI2 and ZK 36374. Representative concentration-response curves are shown in Figure G.3.

The concentration-response curve of PGI2 was not parallel to that of PGE2, being flatter and covering a larger concentration range. PGI2 gave a range of 83-107% of the relative maximum response (PGE2=100%) with a mean value of 99 ± s.e.2.4 (n=10). PGI2 elicited threshold response (tension change of about 5% maximum response) at a mean

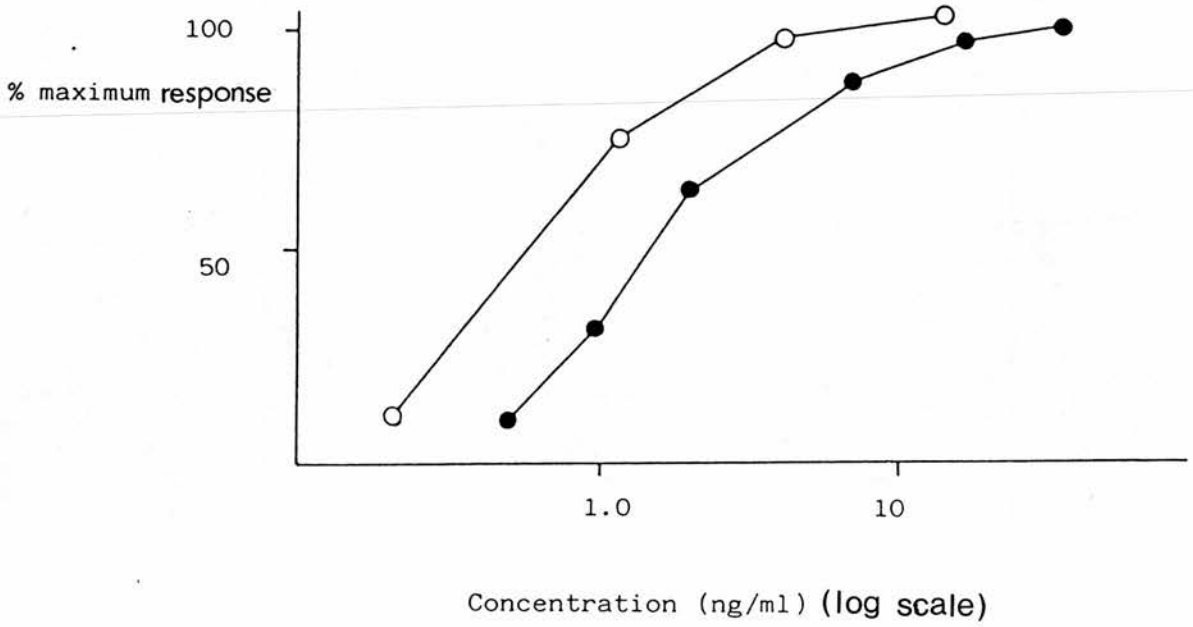


Figure G.2a Rat gastric fundus preparation: cumulative concentration-response relationships for PGE_2 (solid circle) and 16,16-dimethyl PGE_2 (open circle).

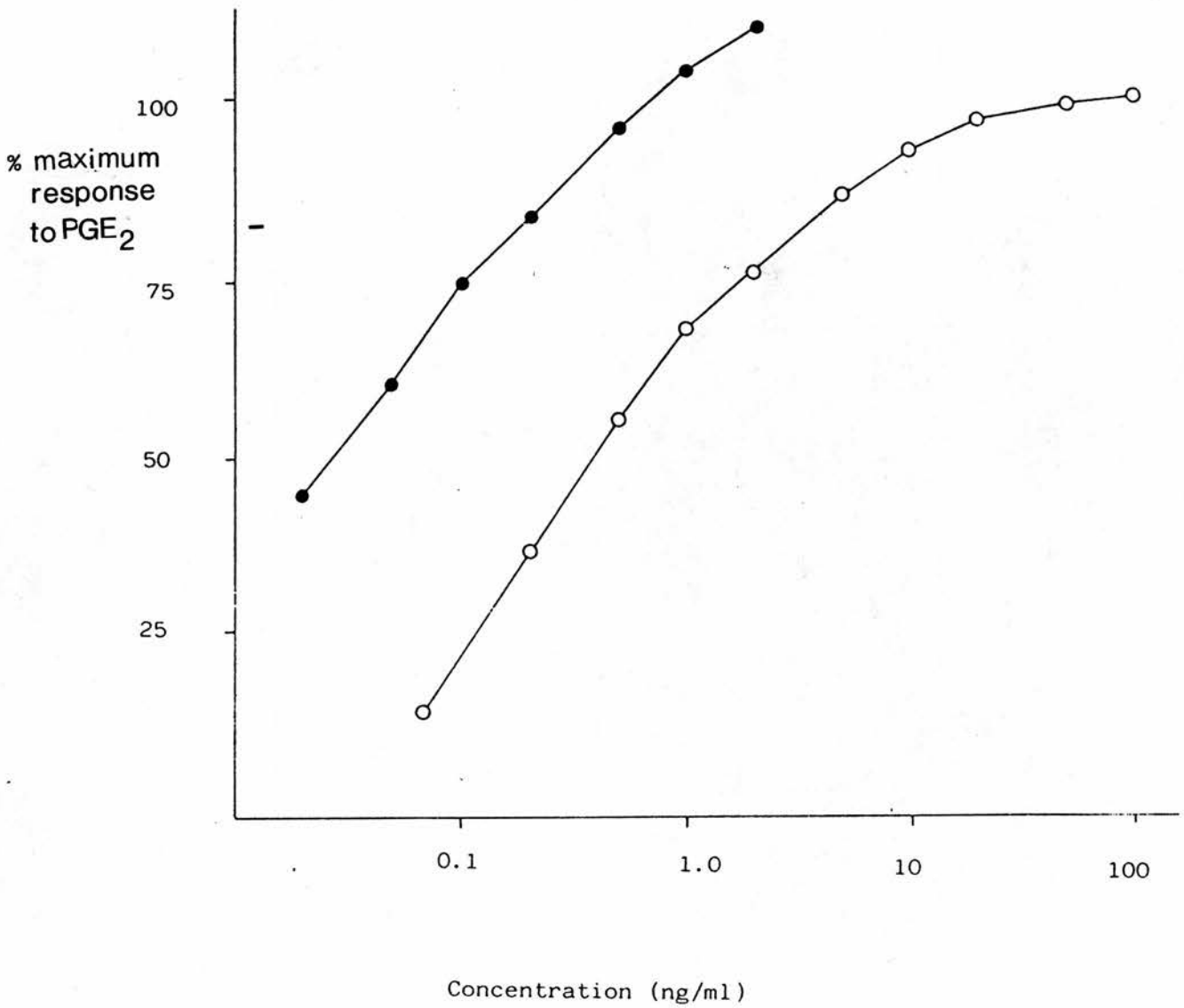


Figure G.2b Rat gastric fundus preparation: cumulative concentration-response relationships for PGE_2 (open circle) and ICI 80205 (solid circle).

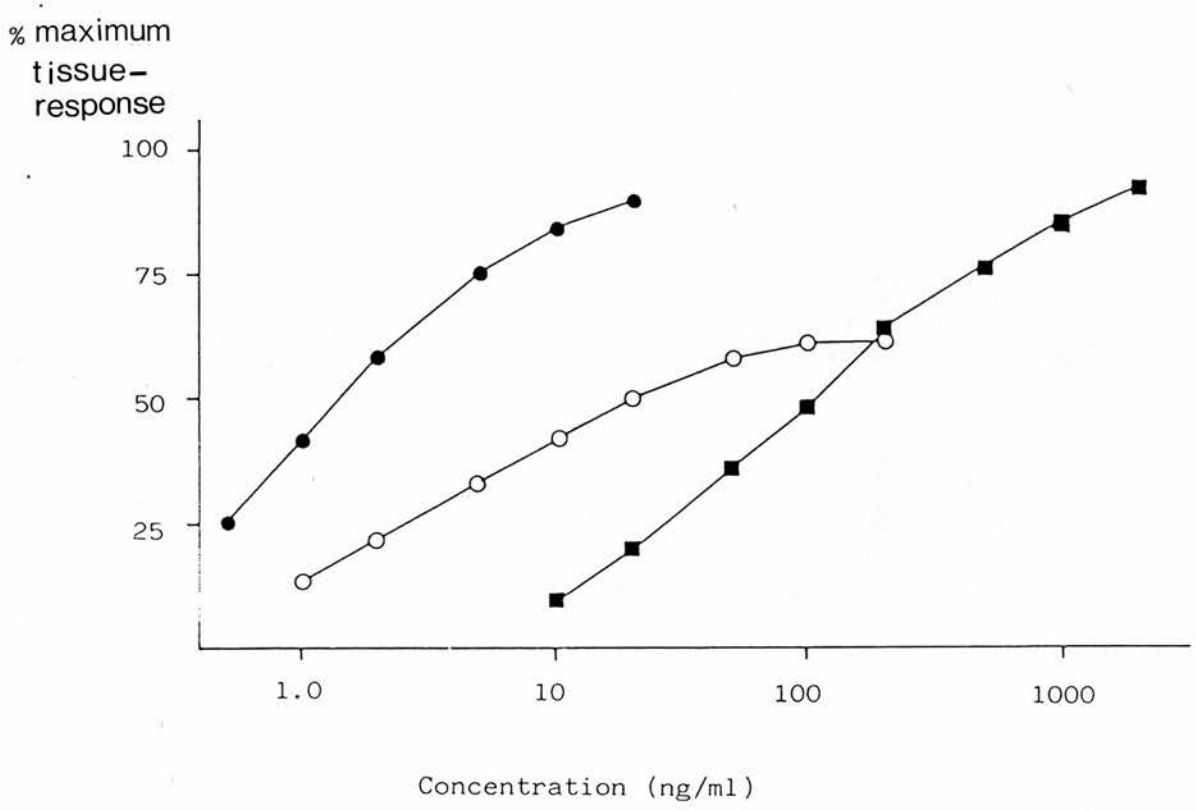


Figure G.3 Rat gastric fundus preparation: log concentration-response curves for PGE_2 (solid circle), ZK 36374 (open circle) and PGI_2 (solid square).

concentration of 7.6 ± s.d.5.1 ng/ml (n=10. range 2.0-20 ng/ml) and 50% maximum response at a mean concentration of 79 ± s.d.55 ng/ml (n=10, range 38-210 ng/ml). PGI2 was about 65 times less active than PGE2. On the other hand ZK 36374 was more active than PGI2 in the preparation: threshold responses were seen with a mean concentration of 0.93 ± s.d.0.32 (n=13. range 2.7-13 ng/ml) (see Table G.3).

On most preparations. 13 out of 15. ZK 36374 produced less than 80% of the relative maximum response (PGE2=100%, mean + s.e.=69 ± 2.7%, n=13, range 53-80%). On the two other preparations both PGE2 and PGI2 showed high activity: EC50 values for PGE2 were 0.5 ng/ml and for PGI2 were 38 and 44 ng/ml. On these preparations ZK 36374 produced a maximum responses which were 100% and 93% of the PGE2 maximum and was 11 and 12 times less potent than PGE2.

The interaction of ZK 36374 with PGE2, PGF2a, 11,9-epoxymethano PGH2 and carbachol was also studied on some of the 13 preparations. In the presence of a fixed concentration of ZK 36374 (200 or 300 ng/ml) cumulative doses of one of the full agonists were added to the organ bath. As shown in Figure G.4, ZK 36374 opposed the contractile action of PGE2. The contractile action of PGF2a, 11.9-epoxymethano PGH2 and carbachol was additive with that of ZK 36374. This further confirmed that ZK 36374 is a partial agonist at the PGE2 receptor site mediating contraction.

Estimates of the affinity constant of ZK 36374 were made using the same method as in the bullock iris sphincter

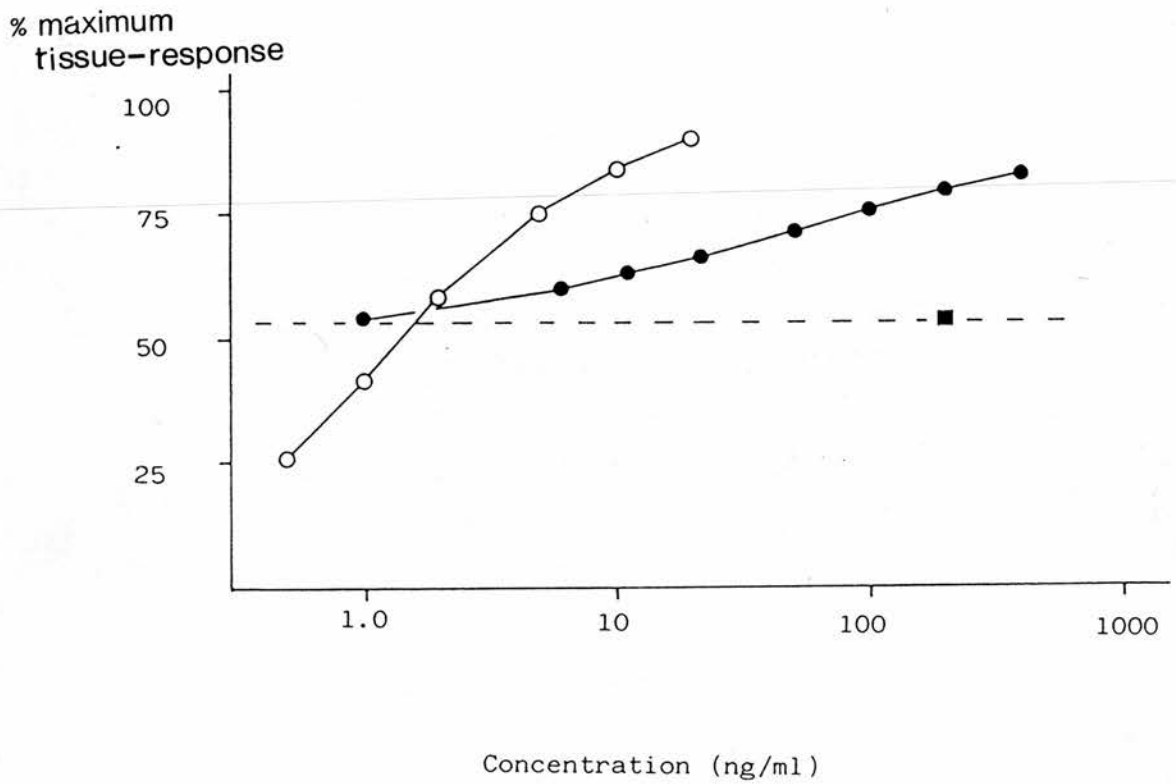


Figure G.4 Rat gastric fundus preparation: interaction of ZK 36374 with PGE_2. A cumulative concentration-response relationship was first established to PGE_2 (open circle) alone, followed by a cumulative relationship to PGE_2 (solid circle) in the presence of a fixed concentration of 200 ng/ml ZK 36374 (solid square).

preparation. From 9 preparations the mean affinity constant was estimated to be 8.3 (± s.e.1.5)x10^{7} M^{-1}.

PGI2 analogues were tested in the presence and absence of TxA2 receptor antagonists to see if any TxA2-like activity could be detected. The procedure was to add a TxA2 receptor antagonist at a certain concentration into the organ bath 10 min before the start of first agonist sequence (PGE2) and to add the same dose of the antagonist 10 min before the start of the second agonist sequence (PGI2 or ZK 36374). The results are shown in Table G.3. EP 116 at 500 ng/ml had little effect on the PGI2 maximum response. On two preparations ZK 36374 produced low maximum responses, 32% and 49% relative to PGE2 and opposed the response to PGE2. The affinity constants are 2.3x10^{8} M^{-1} and 8.3x10^{7} M^{-1}, respectively. On two other preparations ZK 36374 produced a high maximum (91% and 95% relative to PGE2) and was 6 and 8 times less active than PGE2. More experiments would be required to determine whether EP 045 produces a significant effect on responses to ZK 36374, since the maximum response to ZK 36374 varied a lot with preparations.

PGF2a Analogues

PGF2a is about 7 times less active than PGE2 on the rat gastric fundus. Its mean threshold concentration is 1.2 ng/ml (± s.d.0.44, n=17. range 0.6-2.0) and EC50 mean value is 11 ng/ml (± s.d.4.7, n=17, range 5.0-22). The maximum response of PGF2a relative to that of PGE2 was variable. On 5 out of 10 preparations, PGF2a produced slightly higher maximum and a steeper response than PGE2 (Figure

Table G.3 Activities of PGI_2 analogues on the rat gastric fundus in the presence and absence of TxA_2 antagonists

Compound	Threshold concentration mean±s.d.	EC_{50} mean±s.d.	Maximum response (%PGE_2 maximum response) mean±s.e.	EPMR (PGE_2=1.0) mean±s.e.
PGI_2	7.6±5.1 (n=10)	79±55 (n=10)	99±2.4 (n=10)	65±12 (n=10)
ZK 36374	0.93±0.32 (n=13)	7.5±3.5 (n=13)	72±3.4 (n=15)	see text
PGI_2 in the presence of EP 116 500 ng/ml	2.0 3.0	40 40	88% 100%	50 86
ZK 36374 in the presence of EP 045 500 ng/ml	0.75±0.38 (n=4)	6.3±3.3 (n=4)	67±16% (n=4)	see text

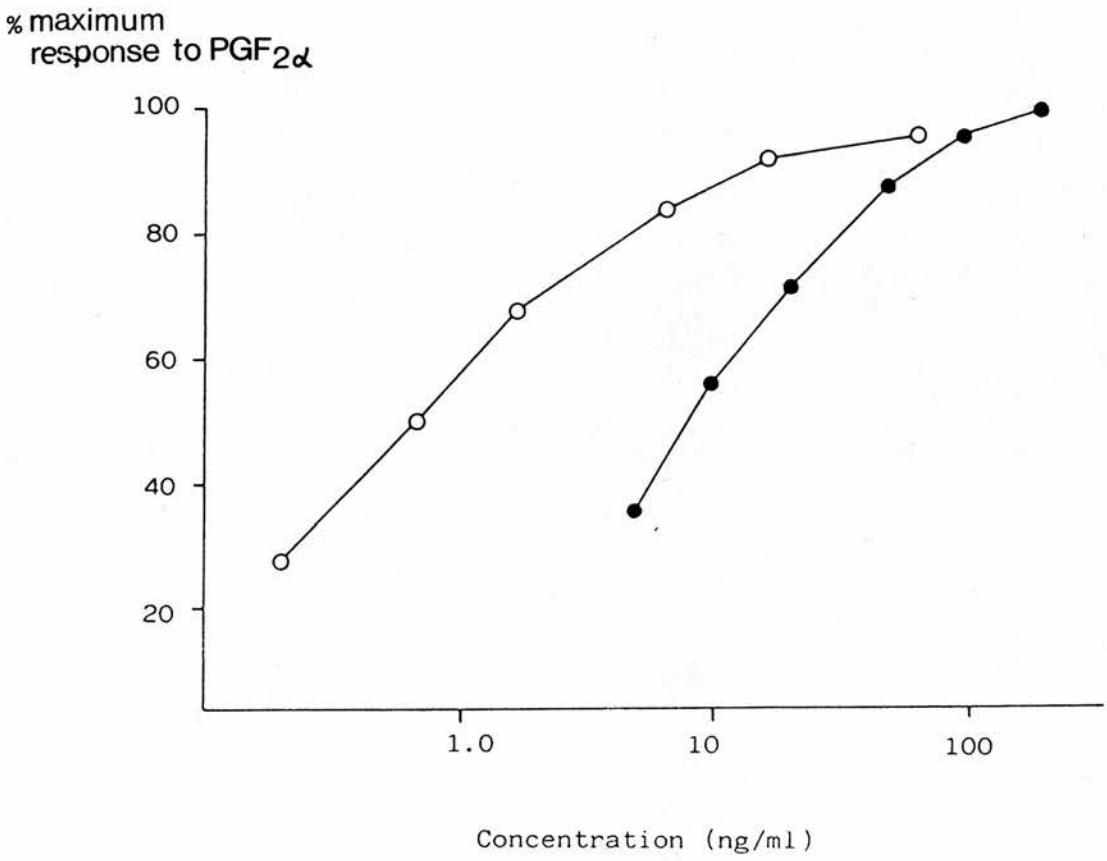


Figure G.5 Rat gastric fundus preparation: cumulative concentration-response relationships for PGE_2 (open circle) and $PGF_{2\alpha}$ (solid circle).

G.5) and on the other 5 preparation PGF2a showed the same maximum as PGE2.

ICI 81008 was more potent than PGF2a and produced a maximum response lower than that obtained with PGF2a (mean ± s.e.=71 ± 8.1%, range 48-85%, n=4) (Figure G.6). Its EC50 values were 0.35, 0.65, 0.85 and 1.0 ng/ml (mean=0.71 ± s.e.0.14 ng/ml, n=4). The interaction of ICI 81008 with PGE2 and PGF2a was studied.

Figure G.7a and 7b show that the action of both PGE2 and PGF2a is additive with that of ICI 81008. In order to exclude the possibility that ICI 81008 may be a partial agonist on the PGF2a receptor site, experiments were done in which ICI 81008 was added into the organ bath when the preparation was contracted by PGF2a at a concentration producing a response near maximum. A typical result is illustrated in Figure G.8. Apparently, ICI 81008 did not oppose the contraction produced by PGF2a. When preparations were contracted with a concentration of ICI 81008 producing its own maximum response, adding PGE2 and PGF2a gave a further contraction, and PGF2a was 50 times less active than PGE2.

ICI 79939 PGF2a was very active on the preparation, being about 25 times more potent than PGF2a and its concentration-response curve was approximately parallel to that of PGF2a (Figure G.9). The activity of ICI 79939 PGF2a was also studied in the presence of the TxA2 receptor antagonists, EP 116 or EP 045. The results show that EP 045 or EP 116 had little effect on the response to ICI

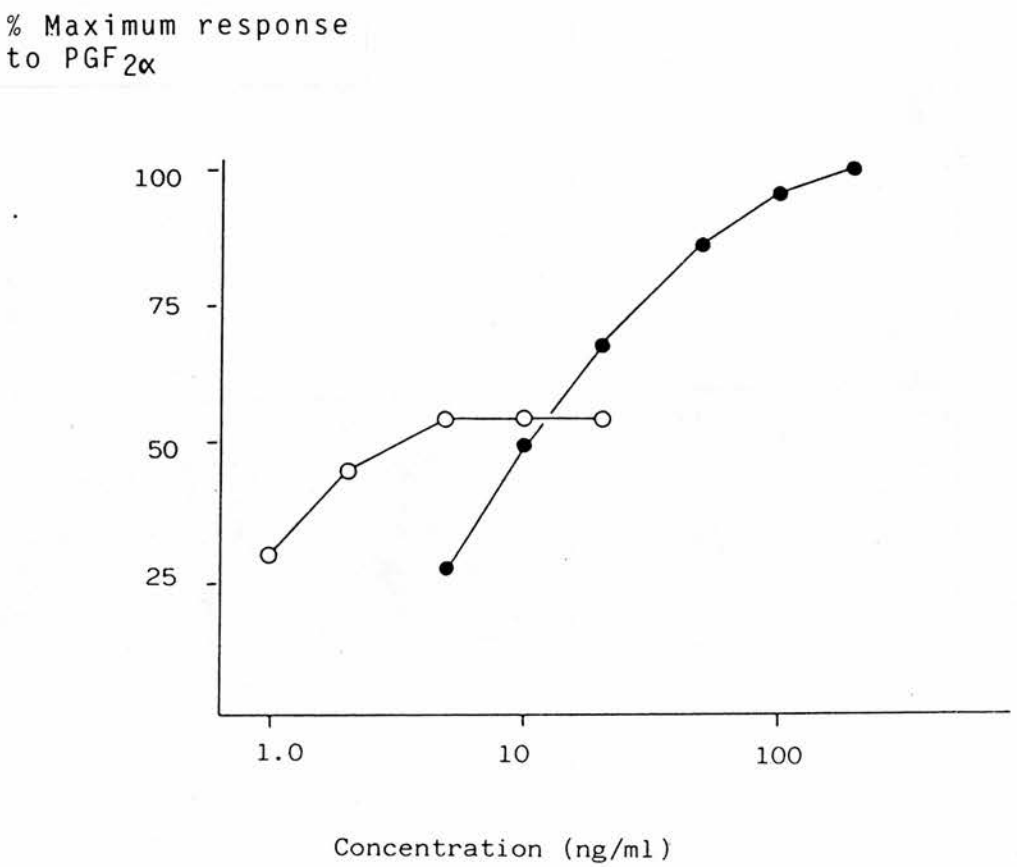


Figure G.6 Rat gastric fundus preparation: cumulative concentration-response relationships for $PGF_{2\alpha}$ (solid circle) and ICI 81008 (open circle).

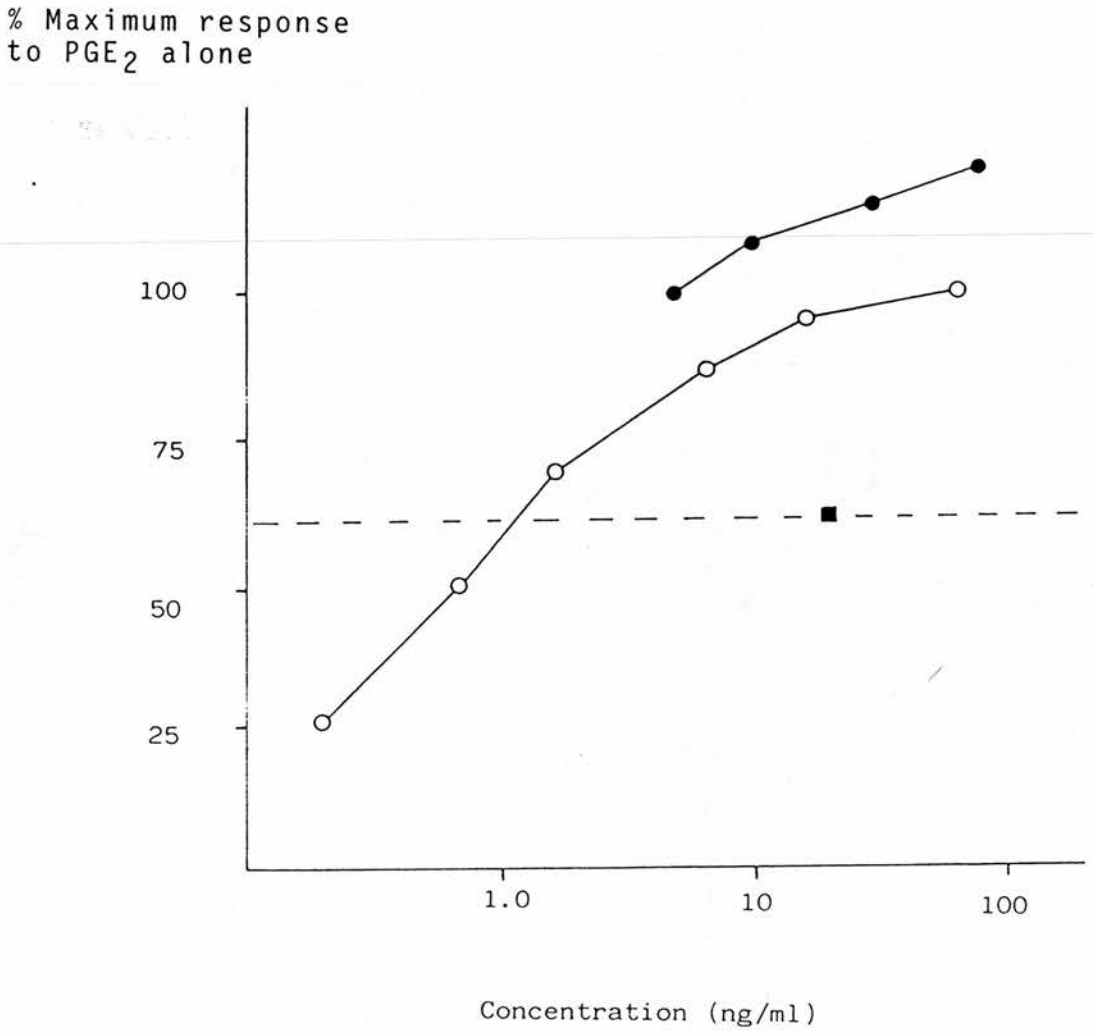


Figure G.7a Rat gastric fundus preparation: interaction of ICI 81008 with PGE_2; log concentration-response curve for PGE_2 acting alone (open circle) and the corresponding curve (solid circle) in the presence of 20 ng/ml ICI 81008 (solid square).

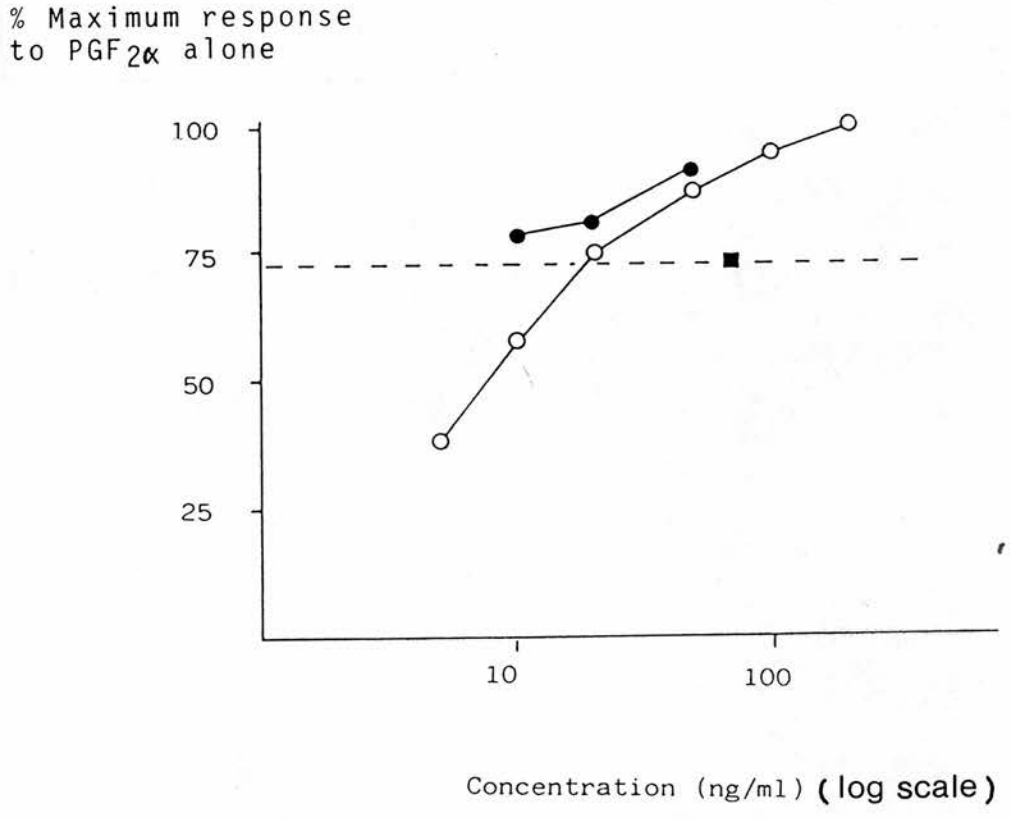


Figure G.7b Rat gastric fundus preparation: interaction of ICI 81008 with $PGF_{2\alpha}$; log concentration-response curve for $PGF_{2\alpha}$ acting alone (open circle) and the corresponding curve (solid circle) in the presence of 70 ng/ml ICI 81008 (solid square).

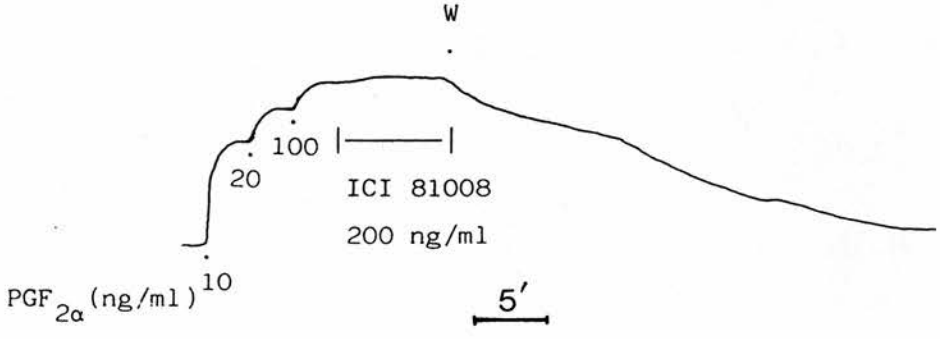


Figure G.8 Rat gastric fundus preparation: interaction of ICI 81008 with $PGF_{2\alpha}$. Adding ICI 81008 does not opposes the response to $PGF_{2\alpha}$. The bathing solution contains indomethacin 10^{-6} M. Changes in tone were recorded isotonically.
W = wash.

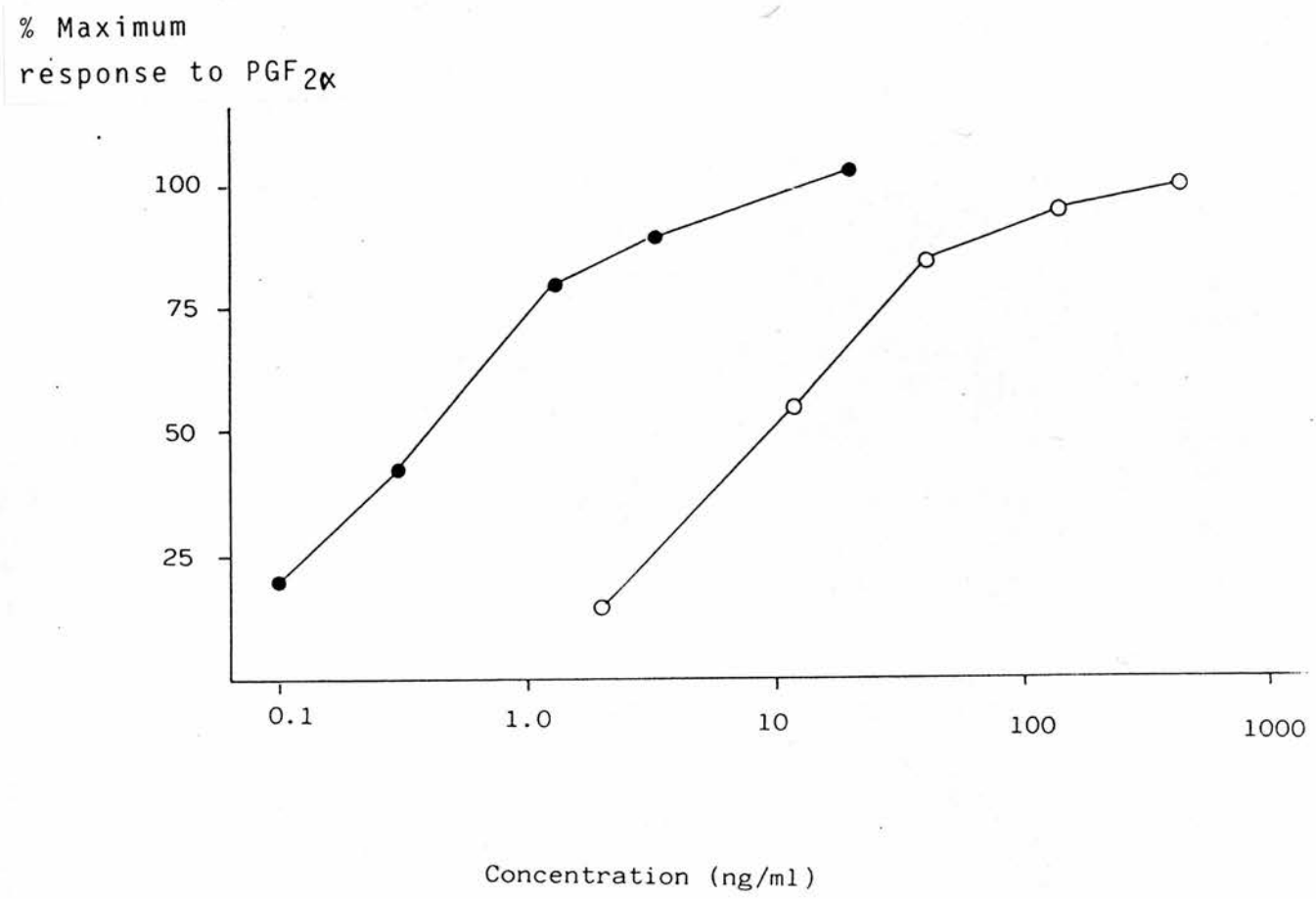


Figure G.9 Rat gastric fundus preparation: log concentration-response curve for $PGF_{2\alpha}$ (open circle) and ICI 79939 $PGF_{2\alpha}$ (solid circle).

79939 PGF2a (Table G.4). PGD2 was tested on two preparations which had been previously treated with high concentrations of both PGF2a (150 ng/ml) and ICI 81008 (200 ng/ml). PGD2 at concentrations 100-1000 times those of PGF2a used initially gave small contractile effects, and PGD2 was about 100 and 240 times less active than PGF2a.

Effects of TxA2 Antagonists

The activity of 11.9-epoxymethano PGH2 is lower than PGE2, but similar to PGF2a. The concentration-response curve of 11.9-epoxymethano PGH2 was steeper and its maximum response greater than that of PGE2 (Figure G.10) on the rat gastric fundus. On 14 preparations threshold concentrations were calculated to be 1.1 $\pm$ s.d.0.66 and EC50 values 9.8 $\pm$ s.d.6.8 ng/ml. EP 045 and EP 116 caused a pronounced shift to the right of the 11,9-epoxymethano PGH2 log concentration-response curve (Table G.5). From the Gaddum-Schild equation were obtained affinity constants of 8.22 $\pm$ s.e.0.91x10^6 M^{-1} for EP 045 at 1.31 μM, 1.5 $\pm$ s.e.0.30x10^8 M^{-1} and 2.1 $\pm$ s.e.0.51x10^8 M^{-1} for EP116 at 45 nM and 90 nM respectively.

Effects of TxA2 antagonists on the responses to PGE2 and PGF2a were studied in two ways: one was to set up a concentration-response relationship of an agonist, add a TxA2 antagonist at a fixed concentration into the organ bath, and 10 min later set up the second concentration-response relationship in the same preparation. After plotting the log concentration-response curves, dose ratios were calculated (see Table G.5). The other way was to

Table G.4 Activities of $PGF_{2\alpha}$ analogues on the rat gastric fundus preparation

Compound	Equipotent molar ratio ($PGF_{2\alpha}$=1.0 with or without TxA_2 antagonists) individual value	number	mean±s.e.
ICI 79939 $PGF_{2\alpha}$	0.030 0.031 0.050	3	0.037± 0.0065
ICI 79939 $PGF_{2\alpha}$ in the presence of EP 045 1μg/ml	0.027 0.039 0.044	3	0.037±0.0050
ICI 79939 $PGF_{2\alpha}$ in the presence of EP 116 500 ng/ml	0.024 0.025 0.031 0.049	4	0.032± 0.0058

Individual values are the result of a comparison with $PGF_{2\alpha}$ on a single preparation. For measuring the potency of ICI 79939 $PGF_{2\alpha}$ in the presence of a TxA2 antagonist experiments were done by adding one of the TxA_2 antagonists into the organ bath 10 min before the start of the first cumulative concentration-response relationship ($PGF_{2\alpha}$) and 10 min before the start of the second cumulative concentration-response relationship (ICI 79939 $PGF_{2\alpha}$). In these cases $PGF_{2\alpha}$ in the presence of a TxA_2 antagonist was taken as standard.

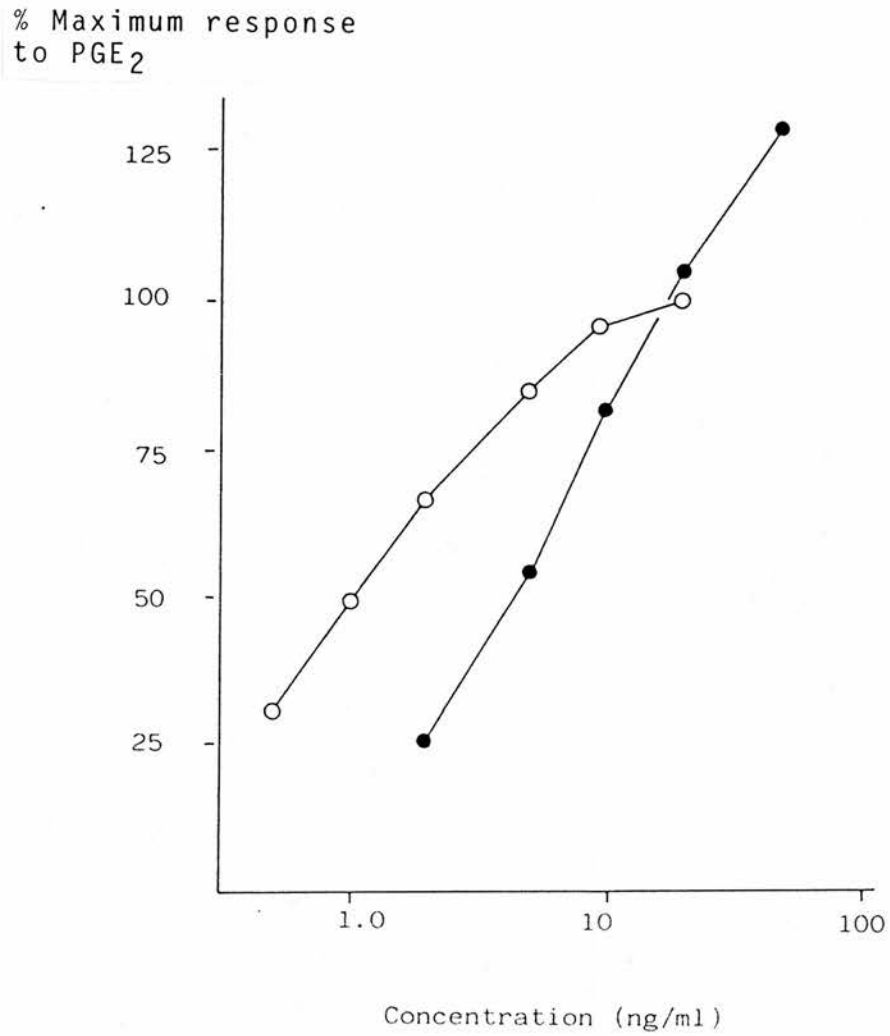


Figure G.10 Rat gastric fundus preparation: log concentration-response curves for PGE_2 (open circle) and 11,9-epoxymethano PGH_2 (solid circle).

Table G.5 Effects of EP 045 and EP 116 on contractile response to different agonists on the rat gastric fundus preparation

Treatment	Agonist	Number of test	Dose-ratios for the second agonist cumulative sequence (first sequence = 1.0)
Control	PGE_2	5	0.74 1.1 1.1 1.1 1.6 (1.1±0.14)
	11,9-epoxymethano PGH_2	2	1.1 1.3
EP 045 1.3 µM	PGE_2	5	0.75 1.3 1.3 1.7 1.9 (1.4±0.20)
	11,9-epoxymethano PGH_2	5	7.7 10 11 12 14 (11±1.0)
EP 116 1.1 µM	PGE_2	4	0.9 1.4 1.6 1.6 (1.4±0.17)
	$PGF_{2\alpha}$	5	1.3 1.8 2.5 2.5 2.6 (2.1±0.25)
EP 116 0.045 µM	11,9-epoxymethano PGH_2	3	5.0 8.5 9.6 (7.7±1.4)
EP 116 0.090 µM *	11,9-epoxymethano PGH_2	3	11 22 26 (20±4.5)

* Values obtained during a third agonist cumulative sequence.

establish the concentration-response relationship of an agonist in the absence of a TxA2 antagonist on one tissue and the concentration-response relationship in the presence of a TxA2 antagonist on another tissue. After plotting the log concentration-response curves, EC50 values were estimated (Table G.6). The two groups of results were consistant with each other. They revealed that EP 045 and EP 116 had little effect on responses to PGE2, but caused a small shift to the right of the PGF2a concentration-response curve.

Other TxA2 Analogues

EP 011, 15-oxo EP 011 and PTA2 were tested. The log concentration-response curve for EP 011 at the concentration less than 10 ng/ml was approximately parallel to that of 11.9-epoxymethano PGH2, while at higher concentration of EP 011 further small contractions could be elicited (Figure G.11). EP 011 was about 20 times more potent than 11.9-epoxymethano PGH2. 15-Oxo EP 011 showed similar potency to that of EP 011. EP 045 at 0.5 μg/ml or EP 116 at 0.5 μg/ml blocked the effects of EP 011 or 15-oxo EP 011.

PTA2 was very weak on this preparation. EP 116 at 0.5 μg/ml and EP 045 at 1.0 μg/ml had a weak effect on the response to PTA2. In the presence of TxA2 antagonists treatment of preparations with PTA2 0.5-1.0 μg/ml for 10-40 min did not oppose the contractile actions of PGE2 and PGI2.

Effect of Verapamil

Verapamil at 5.0 μM shifted the concentration-response

Table G.6 Effects of TxA_2 antagonists on responses to PGE_2 and $PGF_{2\alpha}$ on the rat gastric fundus

Treatment	EC_{50} values (mean±s.e.)	
	PGE_2	$PGF_{2\alpha}$
Control	1.7±0.27 (n=49)	11±1.1 (n=17)
EP 045(1.3 uM)	1.4±0.21 (n=15)	25±9.8 (n=3)
EP 116(1.1 uM)	1.1±0.22 (n=7)	27±4.5 (n=5)

EC_{50} values are obtained from first sequence.

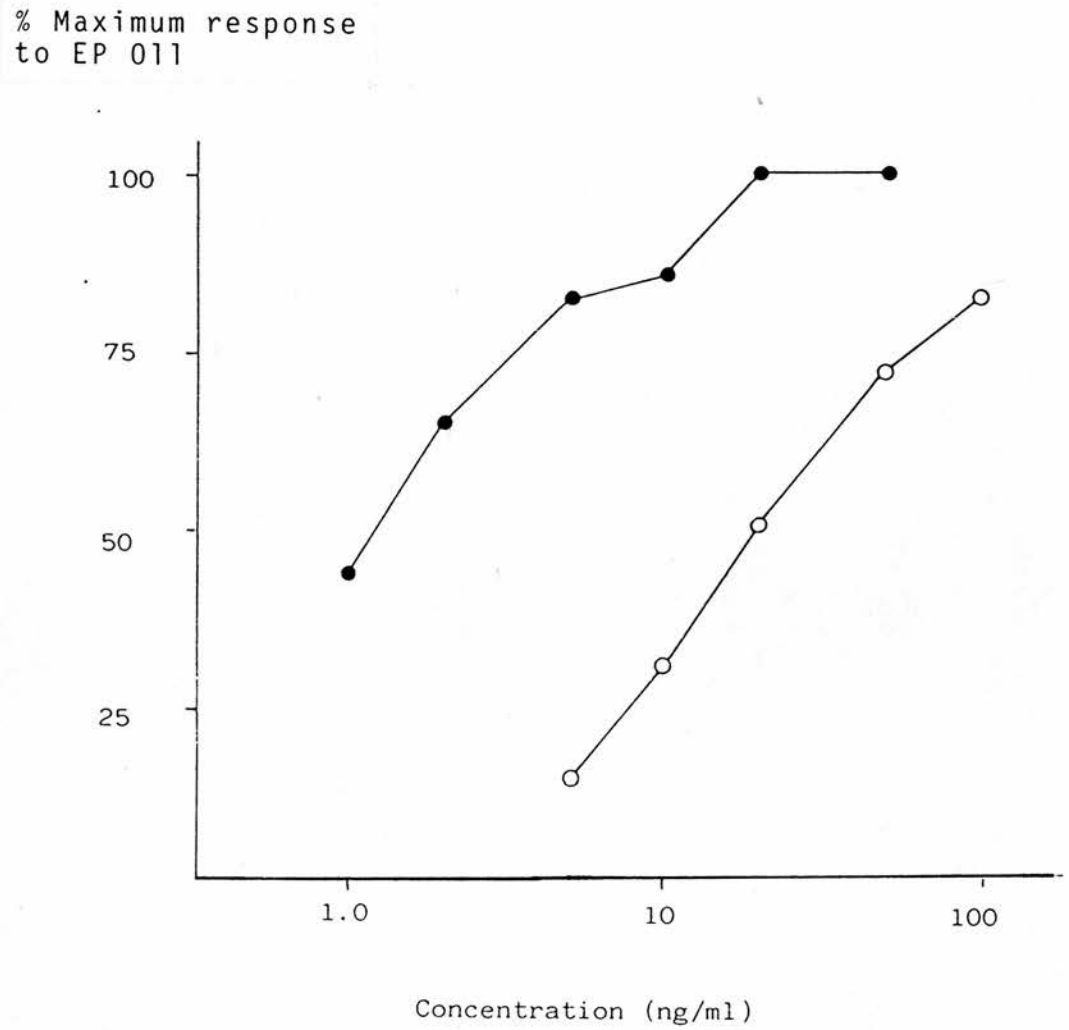


Figure G.11 Rat gastric fundus preparation: log concentration-response curves for 11,9-epoxymethano PGH_2 (open circle) and EP 011 (solid circle).

curves of both 11,9-epoxymethano PGH2 and PGE2 to the right and lowered the maximum response (Figure G.12a and 12b).

Guinea-Pig Ileum Longitudinal Muscle

The guinea-pig ileum longitudinal muscle preparation was suspended in Tyrode's solution containing atropine 0.02 μM since it has been found that prostaglandins can produce contraction partly by stimulating intrinsic cholinergic nerves (Bennett, Eley & Scholes, 1968).

The preparation contracted to PGE2, PGI2, PGF2a and 11,9-epoxymethano PGH2 (PGD2 was not tested). Responses to a fixed concentration of a prostanoid showed considerable variation on the same preparation, when the interval between two single doses was less than 2 min. The interval between two single doses was therefore increased from 2 min to 3-5 min in order to obtain a reproducible response. PGE2 is the most potent of the agonists tested: threshold responses were seen with concentrations of 1.4 ± 0.60 ng/ml (mean ± s.d., n=12) and the EC50 was 20 ± 11 ng/ml (mean ± s.d., n=12). The effects of PGE2 were not influenced by indomethacin (1.0 μM).

PGI2 and ZK 36374 were fairly active but showed irregularly shaped log concentration-response curves. It was therefore difficult to measure their potency relative to PGE2. Figure G.13 shows typical concentration-response curves for PGE2, PGI2 and ZK 36374.

PGF2a and 11,9-epoxymethano PGH2 were weak contractile

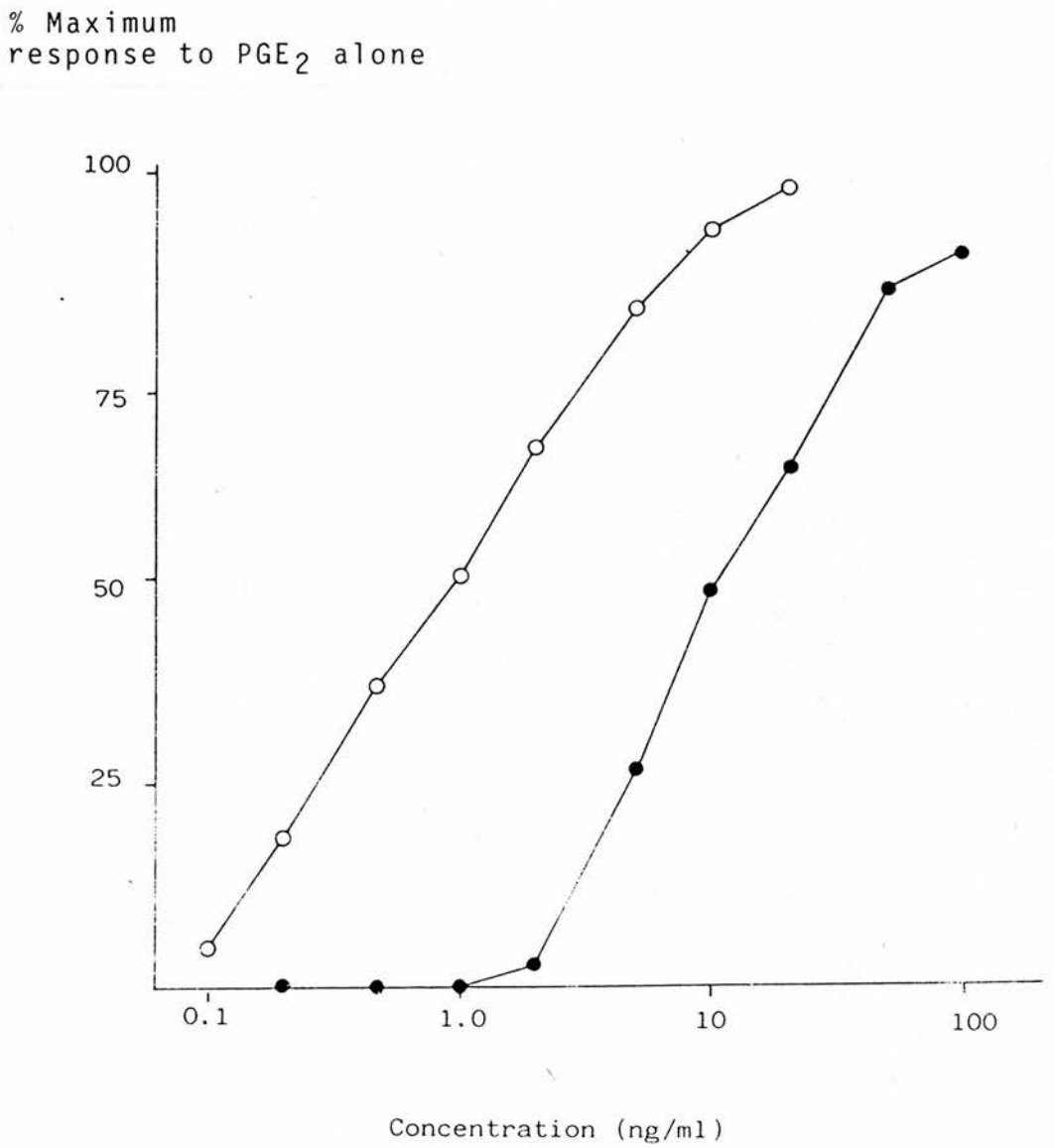


Figure G.12a Rat gastric fundus preparation: inhibitory effects of verapamil on the contractile response to PGE_2. A cumulative concentration-response relationship was first established to PGE_2 (open circle), followed by a cumulative relationship (solid circle) in the presence of a fixed concentration of 5×10^{-5} M verapamil.

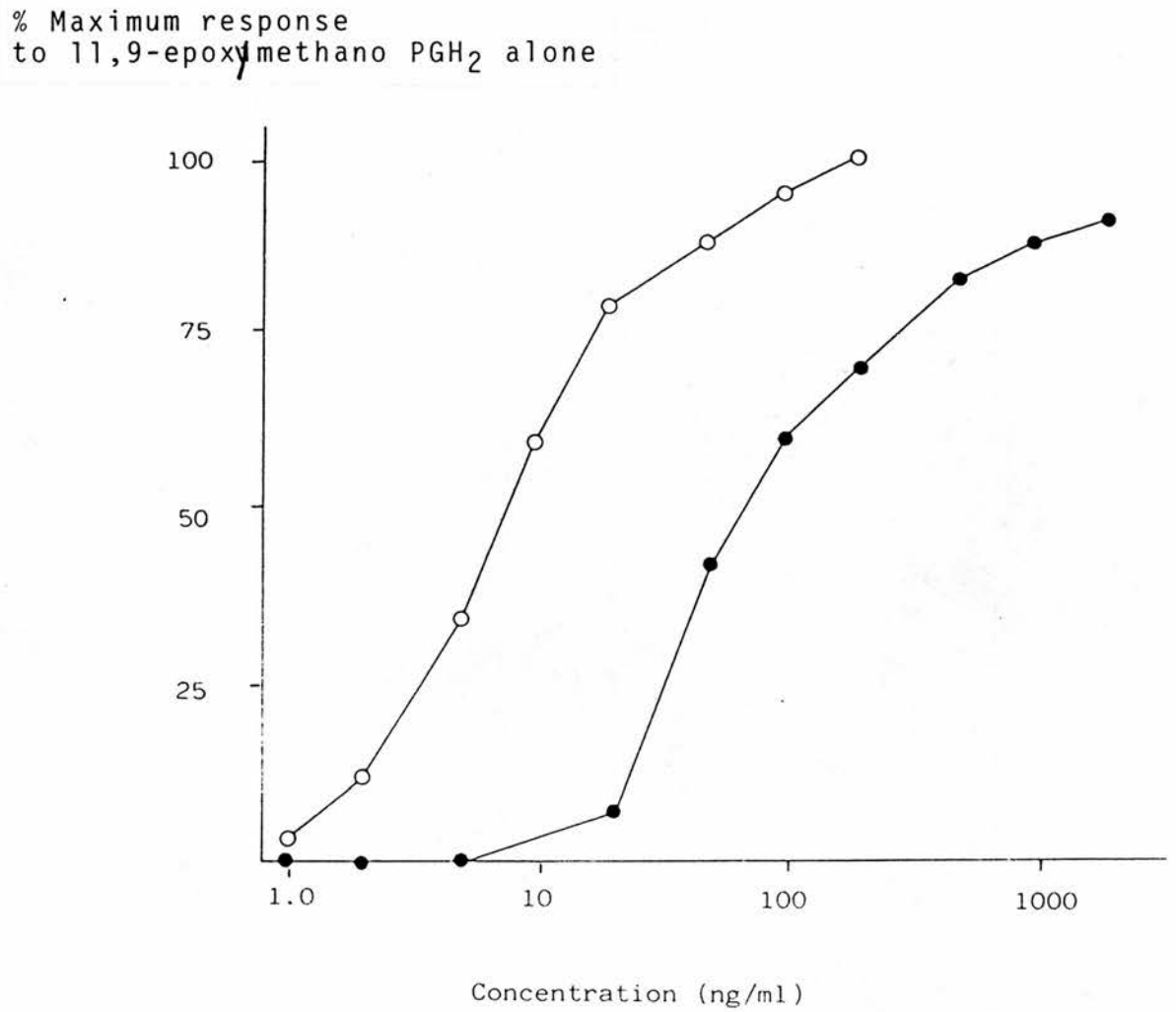


Figure G.12b Rat gastric fundus preparation: inhibitory effects of verapamil on the contractile response to 11,9-epoxymethano PGH_2. A cumulative concentration-response relationship was first established to 11,9-epoxymethano PGH_2 (open circle), followed by a cumulative relationship (solid circle) in the presence of a fixed concentration of 5×10^{-5} M verapamil.

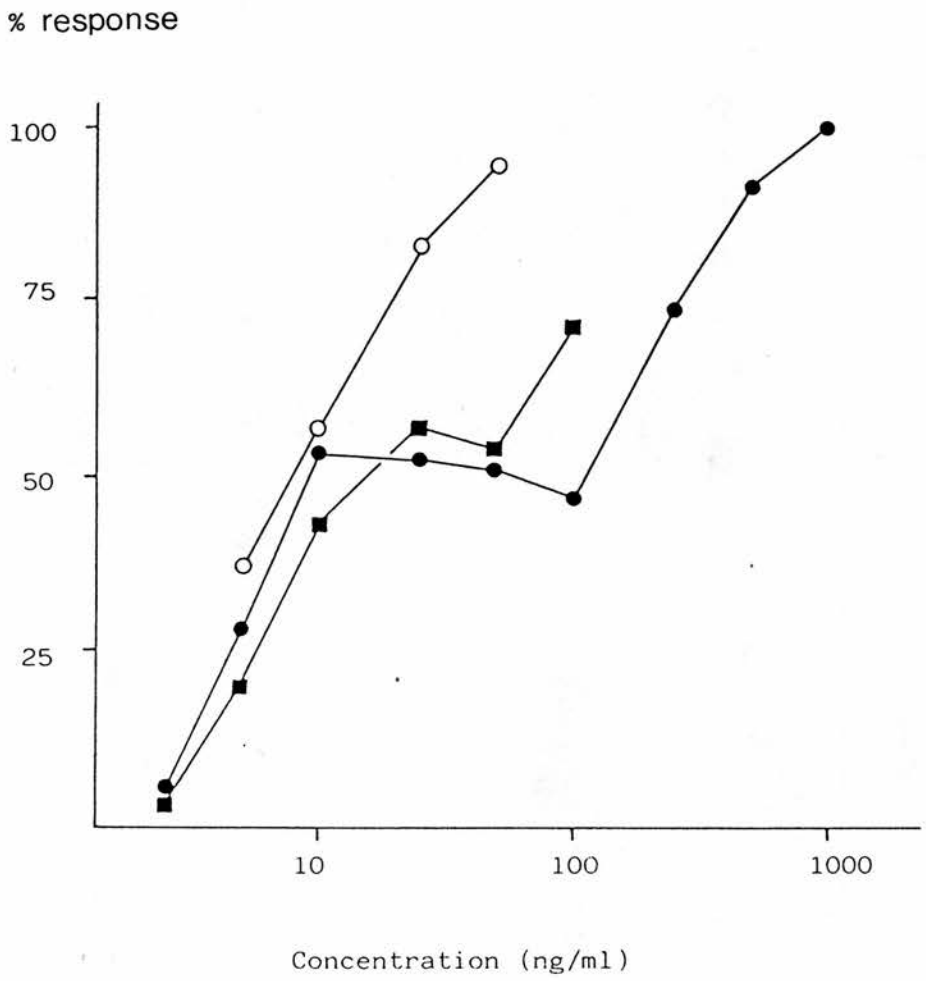


Figure G.13 Guinea-pig ileum preparation: concentration-response relationships for PGE_2 (open circle), PGI_2 (solid circle) and ZK 36374 (solid square).

agents. At 100 ng/ml they produced small contractile effects.

Effects of Cyproheptadine, Methysergide, Dibenamine and Mepyramine

Cyproheptadine (a 5-HT and histamine antagonist), methysergide (a 5-HT antagonist), dibenamine (an alpha-adrenergic blocker), and mepyramine (a histamine antagonist) at 1 μM for each compound, were studied in order to see whether they have any effects on responses to prostaglandins. Fixed submaximum concentrations of PGE2 (10 ng/ml) and PGI2 (50 or 100 ng/ml) were chosen. The experiments in the absence and presence of one of these antagonists were conducted on a single preparation in the following way: a fixed concentration of PGE2 or PGI2 was added into the organ bath, and left until the response reached its plateau. Following 2 or 3 wash-outs, a fixed concentration of one of the antagonists was added and allowed 3 to 5 min contact with the preparation, then the same amount of PGE2 or PGI2 was added. Responses were counted as the height (mm) of clear rise above the basic tone level. The results are shown in Table G.7. Cyproheptadine significantly reduced the contractile effects of both PGE2 and PGI2. Methysergide inhibited responses to PGI2, but had no effect on responses to PGE2. In certain cases methysergide caused a contraction. This may be due to its partial agonist activity on 5-HT receptor sites (Apperley, Feniuk, Humphrey & Levy, 1980). Mepyramine seemed to enhance the action of PGE2.

Table G.7 Effects of some neurotransmitter receptor antagonists on the response to PGE_2 and PGI_2 on the guinea-pig ileum preparation.

Treatment	Increase in tone (mean±s.e.) (mm) PGE_2	PGI_2
Control	22±2.9 (n=12)	20±2.1 (n=8)
Cyproheptadine	13±2.4 (n=12)	5.4±1.4 (n=8)
Control	28±2.2 (n=6)	26±2.5 (n=7)
Methysergide	28±3.3 (n=6)	16±3.5 (n=7)
Control	26±6.9 (n=5)	17±6.6 (n=3)
Dibenamine	22±5.5 (n=5)	11±4.9 (n=3)
Control	23 21	17 17
Mepyramine	30 27	16 17

Guinea-Pig Colon

All the naturally-occurring prostanoids and also 11,9-epoxymethano PGH2 contracted both guinea-pig colon longitudinal muscle and circular muscle preparations. Responses to the prostanoids were elicited rapidly, reaching a maximum in 2-3 s, then fading. 11,9-Epoxymethano PGH2 was very potent on the circular muscle. PGF2a and PGD2 had similar activities on both the longitudinal and circular muscle preparation.

Rat Colon Longitudinal Muscle

These experiments was carried out to compare the activity of PGF2a with that of 15 α EP 130, since it is known that the preparation is very sensitive to PGF2a rather than other prostanoids.

The preparation has spontaneous phasic activity and responded quickly to PGF2a. The contractile response started 2-3 s, reached a maximum in 10-20 s, and relaxed spontaneously. The contractile action of 15 α EP 130 was slow in on-set and off-set. Responses to 15 α EP 130 peaked after 2-4 min and were sustained. The response was accompanied by a rise in phasic activity. which could be inhibited by atropine (2 μM). Following wash-out the response returned to the basic tone level gradually. Concentration-response curves for PGF2a and 15 α EP 130 are shown in Figure G.14.

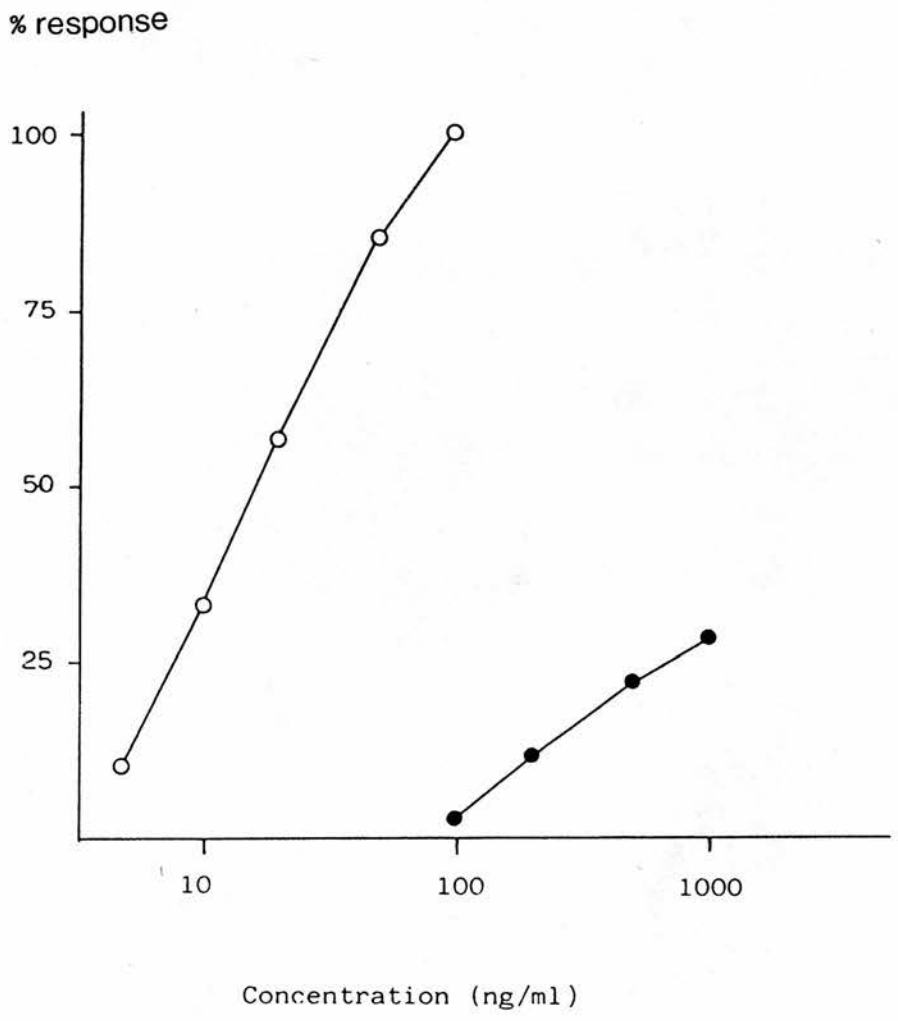


Figure G.14 Rat colon preparation: concentration-response curve for $PGF_{2\alpha}$ (open circle) and 15 α EP 130 (solid circle).

Threshold concentrations for PGF2a ranged from 0.5 to 3.0 ng/ml and EC50 values were 7.5-20 ng/ml. On the other hand, 15 α EP 130 produced only 27-40% of the maximum response obtainable with PGF2a. Its threshold concentration was less than 100 ng/ml, and its EC50 values had a range of 100-300 ng/ml. The interaction of 15 α EP 130 with PGF2a was also studied. A fixed concentration (1μg/ml) of 15 α EP 130 was added into the organ bath first. Between 2-6 min later when the response to 15 α EP 130 had reached its plateau, a dose of PGF2a was added. A typical result is shown in Figure G.15. The contractile effect of PGF2a was opposed by 15 α EP 130.

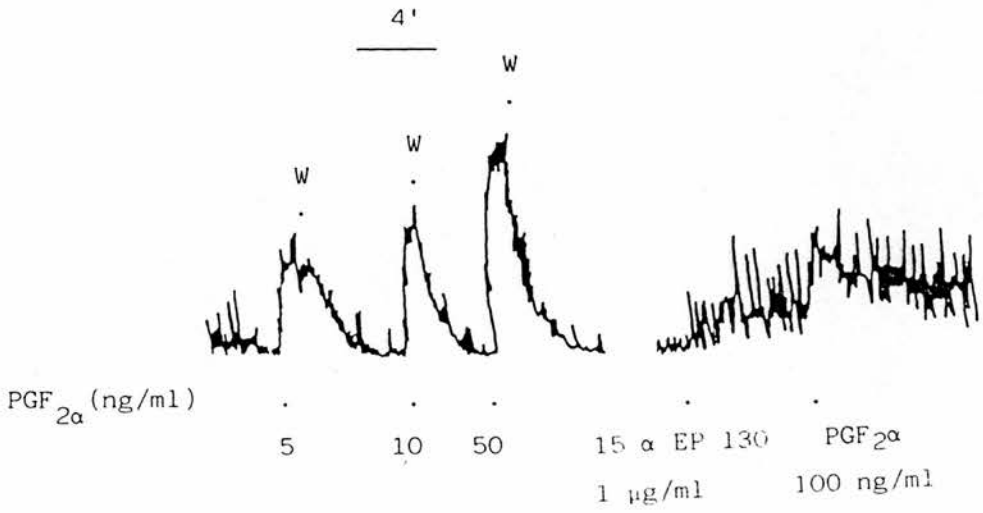


Figure G.15 Rat colon preparation: interaction of 15 α EP 130 with $PGF_{2\alpha}$. The figure shows 15 α EP 130 at 1 µg/ml opposes the contractile action of $PGF_{2\alpha}$.

DISCUSSION

Rat Gastric Fundus Longitudinal Muscle

In agreement with Bennett, Jarosik, Sanger and Wilson (1980), we have found that the rat gastric fundus contains PGE2 and TxA2 receptors.

The TxA2 receptor in this preparation has similar properties to those in the bullock iris sphincter, the dog saphenous vein and the guinea-pig trachea. In particular, the contractile activity of 11,9-epoxymethano PGH2 on the gastric fundus longitudinal muscle is blocked by TxA2 receptor antagonists EP 045 and EP 116. Affinity constants of 8.2×10^{6} M^{-1} for EP 045 and 1.8×10^{8} M^{-1} for EP 116 (see Table E.12) are similar to values obtained on the above three preparations. Furthermore, EP 011 and 15-oxo EP 011 show high activity, 20 times more active than 11,9-epoxymethano PGH2, a result similar to that in the bullock iris sphincter preparation. The further contraction induced by EP 011 at higher concentration can be attributed to its PGE2 or/and PGF2a activity (see below). Because of the existence of TxA2 receptors in the fundus preparation, TxA2 receptor antagonists (EP analogues) were widely used in this study when investigating other PG receptors. The other compound which is widely quoted as being a thromboxane receptor antagonist is PTA2. PTA2 opposed contraction of the rat fundus elicited by azo or epoxymethano analogues of

PGH2, but it showed weak contractile activity which varied with the preparation used. Nicolaou, Magolda, Smith, Aharony, Smith and Lefer (1979), and Aharony, Smith, Smith, Lefer, Magolda and Nicolaou (1980) found that PTA2 potently antagonized constriction of cat coronary artery and aggregation of human platelets induced by PGH2 analogues. Ansell, Caton, Palfreyman, Stuttle, Tuffin and Walker (1980) obtained a slight stimulation of rabbit aorta and mesenteric artery, and a weak antagonism of the TxA2-like material released from guinea-pig lung. More recently, Jones, Peesapati and Wilson (1982) showed that PTA2 was a partial agonist on the TxA2 receptor site in rabbit aorta, dog saphenous vein and guinea-pig trachea preparations. PTA2 caused shape change but not aggregation in washed human platelets (Jones, Personal Communication). Bennet and Sanger (1982) have also examined the action of PTA2 on the rat fundus. They found that PTA2 (0.5 µg/ml) antagonized submaximal contractions of the rat gastric fundus to 11,9-epoxymethano PGH2. However, they claimed that the effect was not selective, since contractions to PGE2, PGF2a, PGI2 and a lesser extent acetylcholine (Ach), were also reduced. Their findings with the longitudinal muscle of human stomach were similar to those with the rat gastric fundus, except that PGI2 relaxes the human preparation and this response was unaffected by PTA2. In contrast, we have found PTA2 did not oppose the effect of either PGE2 or PGI2 on the rat gastric fundus or the effect of PGF2a on the dog iris sphincter. The discrepancy may be due to two factors: (1) we used indomethacin to inhibit endogenous biosynthesis

of PGs; (2) in our case the preincubation time with PTA2 was 10-40 min, in their experiments 1 h was chosen. We also used a higher concentration (1 µg/ml) of PTA2. One can speculate that PTA2 may inhibit endogenous production of PGs stimulated by exogenous PGs (this factor was eliminated by using indomethacin in our study), or when a prolonged period of preincubation is adopted PTA2 may affect events in the excitation-coupling chain, since PTA2 also depresses responses to Ach. In our hands PTA2 appears to exert a specific action at the TxA receptor in the rat fundus. It may be a partial agonist rather than a true antagonist.

The isolated rat gastric fundus has commonly been employed for the detection and assay of PGE-like substances in biological fluids. Our results show that the PGE2-sensitive system responds to PGE2, 16,16-dimethyl PGE2 and ICI 80205. The order of potency is ICI 80205> 16,16-dimethyl PGE2> PGE2, consistent with the results from the bullock iris sphincter experiments. ICI 80205 and 16,16-dimethyl PGE2 are slightly more active in this preparation than in the iris sphincter relative to PGE2. This is not due to their TxA2-like activity since TxA2 receptor antagonists do not affect their potency and maximum response. The additional contraction elicited by higher concentrations of ICI 80205 may be caused by an action on PGF2a receptor sites (see later).

In addition to PGE2 and TxA2 receptor sites, we have shown that a PGF2a-sensitive system may exist in the gastric fundus. It is characterized by the high activity of

ICI 81008, a specific PGF2a-like agonist. ICI 81008 is about 15 times more potent than PGF2a. Its lower maximum response relative to that of PGF2a is probably due to its inactivity on PGE2 receptor sites, since we have shown on the bullock iris sphincter that ICI 81008 is a very weak agonist, but PGF2a is capable of acting on the PGE2 receptor system (the EPMR is about 50, PGE2=1.0). Since the rat fundus is highly responsive to PGE2 one can expect that PGF2a will interact with the PGE2 receptor to produce contraction. The possibility that ICI 81008 might be a partial agonist on the PGF2a receptor was also considered. This can be excluded since the response to PGF2a was not opposed by ICI 81008 on the rat gastric fundus. It is relevant that on the dog and cat iris sphincter muscle preparations ICI 81008 is a full agonist on the PGF2a receptor site. Another possibility is that there may be two subtypes of PGF2a receptors in the rat gastric fundus: ICI 81008 is a full agonist on one receptor site and an inactive or an antagonistic agent on the other. As we have demonstrated, in the presence of ICI 81008 at a concentration which gave a maximum response, PGF2a elicited additional contractions at concentrations about 50 times greater than PGE2, similar to the relative potency of PGF2a on PGE2-sensitive system of the bullock iris sphincter. This indicates that the additional contraction induced by PGF2a may be due to activation of PGE2 receptor. And up to now, there is no evidence which suggests the existence of two subtypes of PGF2a receptor.

ICI 79939 PGF2a is more active than ICI 81008 in this preparation in contrast to the dog iris sphincter where

ICI 81008 is more active than ICI 79939 PGF2a. The high activity of ICI 79939 PGF2a on the rat gastric fundus can be explained by its great potency on the PGE2 receptor, but not on the TxA2 receptor since its activity is not altered by TxA2 receptor antagonists.

ZK 36374 is more active than PGI2 on the rat gastric fundus. Although, relative to PGE_2, ZK 36374 produces slightly higher maximum responses on the gastric fundus than on the bullock iris sphincter, in most cases it gives a lower maximum response than PGE2, and opposes the response to PGE2, a profile similar to its activity in the bullock iris sphincter. This suggests a partial agonist action on the PGE2 receptor site.

The higher maximum response to ZK 36374 on the gastric fundus could however be explained by the presence of PGI2 receptor in the tissue. This is supported by the finding that PGI2, although it is a much weaker agent, does elicit a higher maximum response in the rat gastric fundus than in the bullock iris sphincter relative to PGE2, and in some cases it gives an even higher maximum response than PGE2. Furthermore, it has been shown that rat gastric fundus microsomes can generate PGI2 (Gryglewski, Bunting, Moncada, Flower & Vane, 1976); this seems to imply that the rat gastric fundus has PGI2 receptor sites. It is unlikely that ZK 36374 and PGI2 act on TxA2 or PGF2a receptor sites, since (1) their actions on the rat gastric fundus are not blocked by TxA2 receptor antagonists, and (2) both PGI2 and ZK 36374 are very weak on PGF2a receptor sites as shown in the dog and cat iris sphincter studies. An alternative explanation

is that the rat gastric fundus has a larger PGE2 receptor reserve than the bullock iris sphincter, which allows ZK 36374 and PGI2 to give higher maximum responses.

Bennett, Jarosik, Sanger and Wilson (1980) have found that trimethoquinol (50 ng/ml) reduced contraction of the rat stomach muscle to PGD2, PGF2a, PGI2, 11,9-epoxymethano PGH2 and PGE1 but not PGE2. Therefore, the effect on PGE2 and PGE1 may differ, although both are reported to have similar binding sites in this tissue (Miller & Magee, 1973). Since PGE1 has both PGE2- and PGI2-like activity: a full agonist on the PGE2 receptor and a partial agonist on the PGI2 receptor, the inhibition of PGE1-induced contraction by trimethoquinol could be due to the depression of the PGI2-like activity. However, since there is certain evidence suggesting the existence of PGE1 receptors, and the existence of PGI2 receptor in the rat gastric fundus has not been proved, we had better leave the question open until selective PGI2 or PGE2 antagonists are available. The existence of a PGE1 receptor we will consider in the General Discussion Section.

Bennett and Sanger (1982) showed that PTA2 opposed PGF2a and PGD2 to different extents. Does this suggest the existence of PGD2 receptors? As we have demonstrated on the cat iris sphincter (P39), PGD2 could have PGF2a-like activity; it is about 35 times less active than PGF2a. Bennett, Jarosik, Sanger and Wilson (1980) have shown that PGD2 was about 5 times less active than PGF2a on the rat gastric fundus. However in our study, following prolonged treatment of the preparation with PGF2a and ICI 81008, PGD2

showed an activity 170 times less potent than PGF2a. If there were PGD2 receptor sites, it would appear that PGF2a and ICI 81008 could desensitize them.

Guinea-pig Ileum

The isolated guinea-pig ileum is another preparation which has commonly been used for bioassay of PGs (Eliasson, 1957; Bergstrom, Eliasson, von Euler & Sjovall, 1959; Eliasson, 1959; Anggard & Bergstrom, 1963; Bergstrom & von Euler, 1963; Horton, 1963; Horton & Main, 1963; Anggard, 1966; Horton & Main, 1965; Pike, Kupiecki & Weeks, 1967).

Several studies have demonstrated that PGE series are released from the guinea-pig ileum (Botting & Salzmann, 1974; Botting, 1977; Kadlec, Masek & Seferna, 1978; Yagasaki, Takai & Yanagiya, 1980). In fact PGE2 is the most potent contractile compound among the naturally-occurring PGs. Like PGEs, PGI2 is synthesized by human and animal intestinal tracts (LeDuc & Needleman, 1979; Bennett, Hensby, Sanger & Stanford, 1981; Whittle, 1981) and contracts the guinea-pig ileal longitudinal muscle (Moncada, Gryglewski, Bunting & Vane, 1976a; Sirois, Borgeat & Jeason, 1981). In this study, we have found PGI2 is fairly active, and ZK 36374 showed a higher activity relative to PGE2 in this preparation than in the bullock iris sphincter. This indicates that there may be PGI2 receptor in the guinea-pig ileum preparation.

Intrigued by the finding that the effect of PGs on the

guinea-pig ileum could be inhibited by cholinergic antagonists, we tried some other neurotransmitters receptor antagonists in an attempt to differentiate PGE2 and PGI2 receptors. As shown in Table G.7, methysergide had little effect on response to PGE2, but inhibited response to PGI2, and mepyramine enhanced the action of PGE2 but did not affect response to PGI2. Cyproheptadine and dibenamine inhibited both PGE2 and PGI2, but to slightly different extent.

Cyproheptadine is believed to be a potent antagonist of the actions of 5-HT, and also has antihistamine and antibradykinin activity. It has been found that in addition to its receptor antagonist activities, cyproheptadine inhibits the release of insulin and glucagon from rat pancreas (Joost, Beckmann, Holze, Lenzen, Posner & Hasselblatt, 1976; Richardson, 1976; Donatsch, Lowe, Richardson & Taylor, 1980), reduces the spontaneous contraction of the rat uterus (Sadovsky, Dora, Pfeifer, Polishuk, Rachaminoff & Sulman, 1973) and suppresses mechanical and electrical activity in guinea-pig taenia coli (Lowe, Matthews & Richardson, 1981). These effects are antagonized by high calcium. Cyproheptadine also decreases calcium uptake into strips of guinea-pig taenia coli (Lowe, Matthews & Richardson, 1981). Recently, Riccioppo Neto (1983) has found that cyproheptadine decreases action potential duration and plateau amplitude of the action potentials on canine cardiac Purkinje and ventricular muscle fibres; these effects were readily antagonized by increase in the extracellular calcium concentration. The effect of

cyproheptadine on action potential characteristics of Purkinje fibres is similar to those described for verapamil and D 600 (Kohlhardt, Bauer, Krause & Fleckenstein, 1972; Cranefield, Aronson & Wit, 1974; Rosen, Ilvento, Gelband & Merker, 1974). He suggests that cyproheptadine reduces inward calcium current via the slow channel, and at higher concentration cyproheptadine might also be exerting depressant effects on the fast inward sodium current. Furthermore, Ganatra, Dhumal, Bhatt and Sachdev (1979) reported that cyproheptadine non-selectively blocked the responses to PGE1, PGF2a as well as Ach and 5-HT at concentrations from 10 ng to 1 µg/ml in rat stomach strip, rabbit uterus, guinea-pig ileum and rabbit jejunum. All these findings indicate that cyproheptadine may be a calcium channel blocker. Thus, the inhibitory effect of cyproheptadine on the guinea-pig ileum preparation may be associated with its calcium channel blocking activity.

Apperley, Humphrey & Levy (1976) observed that cyproheptadine was weak in blocking noradrenaline-induced contractile response in the rabbit aorta and ear artery. This is probably due to noradrenaline-mobilization of intracellular as well as extracellular calcium pools in those two preparation, as suggested by Deth and van Breemen (1974, 1977), and Manzini, Maggi and Meli (1983).

Dibenamine, a haloalkylamine, has many pharmacological actions in addition to alpha-adrenoceptor blockade. It has antagonistic action against histamine, 5-HT, Ach (muscarinic), angiotensin and bradykinin. Thus, it is hard

to tell through which antagonism dibenamine produces the inhibitory effect on both PGE2 and PGI2. However one can at least say that it is not due to antagonism on histamine receptors since mepyramine did not oppose PGI2 action.

Atropine has an affinity constant of 10^9 M^{-1} for Ach receptor in the guinea-pig ileum. We used the concentration of 0.02 μM. In work by Kennedy, Coleman, Humphrey, Levy & Lumley (1982) atropine at 0.8 μM, 40 times higher than the concentration we used, was chosen. They showed that relative to PGE2, PGI2 was far less potent in their study than in our experiments. This may indicate that PGI2 may be a powerful Ach releasing agent, and the concentration of atropine we chose was not high enough to block all activity of the released Ach. This idea is supported by more recent findings of Gaion and Trento (1983). They have demonstrated that PGI2-induced contractions were inhibited by atropine and potentiated by physostigmine. Tetrodotoxin as well as low temperature abolished and beta-bungarotoxin reduced the effect of PGI2, and in the presence of tetrodotoxin PGI2 failed to affect the response to a sub-maximal concentration of Ach. They suggest that PGI2 releases Ach from intramural nerves possibly by increasing the excitability of cholinergic cell bodies. Since PGI2 was very active (its threshold concentration was less than 1 nM), it is probable that PGI2 receptors exist on the intramural cholinergic nerves of the guinea-pig ileum. It is still unknown whether there are PGI2 receptors in the guinea-pig ileal longitudinal muscle itself. Therefore dibenamine may inhibit the response to PGI2 by antagonising the released Ach, since

it has antagonistic action on the muscarinic receptor.

Guinea-pig Colon

PGD2 is synthesized enzymatically in the gut and the brain of mammals. It has been found that PGD2 has a distinct and interesting profile of biological activity of its own. PGD2 is a potent vasopressor agent in the sheep, being 70 times more potent than PGF2a (Jones, 1976). This action is not blocked by EP 045 (Jones, Personal Communication). PGD2 potently inhibits aggregation of human, sheep and horse platelets, and it has been suggested that PGD2 acts on a different receptor than do PGI2 and PGEl. Recently, Ishizawa and Minowa (1982) have shown that both PGD2 and PGF2a were active in the guinea-pig colonic longitudinal and circular muscle. In particular, the contractile action of PGD2 was more potent than that of PGF2a in the circular muscle. In the hope of finding a PGD2-sensitive preparation to characterize the PGD2 receptor we tried both these preparation. Unfortunately, we have found that these preparations are sensitive to other PGs as well. One thing we need to mention is that PGE2 consistently displayed contractile activity in our study, while Sanger and Bennett (1980) showed that PGE2 relaxed guinea-pig colonic circular muscle, and only in some cases did it cause contraction alone or preceeding the relaxation.

Rat Colonic Longitudinal Muscle

The rat colonic longitudinal muscle is sensitive to PGF2a (Jones, Personal Communication). 15 α EP 130 showed a partial agonist activity in this preparation, which is consistent with our previous results on the dog iris sphincter.

Section Four

Actions of Prostanoids on the Rat Platelets

Reference citations: pp 355-370

INTRODUCTION

Platelets, which are circulating pieces of megakaryocyte cytoplasm are excitable. The most familiar excitable responses are adhesion and aggregation. Both of them are involved in haemostasis and its pathological aberration, thrombosis. The processes of adhesion and aggregation are simplest to quantitate in vitro. Aggregation is presently more frequently studied than adhesion. The most commonly used method for studying the aggregation process is based on the light transmission method first described by Born (1962), and subsequently reviewed by O'Brien (1971), Attar, McLaughlin and Masaitis (1973) and others.

Platelet activation is a sequence of morphological and functional changes, with pseudopod formation, centralisation of the contractile proteins, accompanied by the formation and secretion of a variety of substances. The secretion reaction consists of the release of intragranular materials (5-HT, ATP, ADP, calcium, the various metabolites of arachidonic acid, platelet-activating factor (PAF), lysosomal enzymes and mitogenic substance) to the extracellular medium (Holmsen & Weiss, 1979). The aggregating agents include ADP, adrenaline, collagen, thrombin, PAF, calcium ionophores and prostanoids.

It has been suggested that prostanoids, ADP and PAF can cause platelet aggregation directly and indirectly (by releasing each other) while Ca^{2+} ionophores, thrombin and collagen

aggregate platelets by release of ADP, PAF and prostanoids. Since the concentration of adrenaline required to induce platelet aggregation by itself is greater than that normally found in the circulation, it may act synergistically with other aggregatory agents.

The turbidometric method has been extensively used to observe the aggregatory process in vitro. The addition of an aggregating agent to an opalescent stirred suspension of platelets results in platelet aggregation and an increase in light transmission through the platelet suspension. The activation of platelets can be divided into three steps: shape change, primary wave and secondary wave. A weak stimulus induces only shape change visualized by a small deflexion in the direction opposite to the aggregating wave, indicating a decrease in light transmission. This change reflects an internal transformation. Using electron microscopy it is found the discoid shape of platelets is lost and the cells become irregularly spherical with multiple spiny pseudopods. The circumferential bundle of microtubules and the enclosed organelles shift centrally. The primary wave reflects a loose platelet-platelet attachment. Change in platelet shape and the primary aggregation are reversible. The secondary wave of aggregation occurs with higher concentrations of agonist and represents largely irreversible aggregation mediated by released ADP, PGs and PAF from platelet granules.

Platelet shape change does not require the presence of external calcium ions, since it occurs in the presence of

high external concentrations of a chelating agent EDTA (Born, 1970) or in calcium-free medium (Rink, Smith & Tsien, 1982). This indicates that intracellular calcium may be involved. In fact, shape change induced by ADP is associated with an increase in the internal free calcium concentration, measured spectrophotometrically using the calcium-indicator murexide, though no (45)Ca is taken up from extracellular sources (Le Breton & Feinberg, 1974). By employing low concentrations of chlortetracycline as a fluorescent probe for membrane-bound calcium, a redistribution of calcium away from membrane sites during the shape shange was shown (Le Breton, Dinerstein, Roth & Feinberg, 1976). It is proposed that the local increase in calcium ions restricted to the submembranous region initiates the breakdown of the circumferential ring of microtubules (Borisy, Olmstead, Marcum & Alen, 1974) and causes local activation of the contractile system giving rise to the pseudopods and spikes observed in the course of shape change. For this reason it is assumed that the release of calcium from the internal surface of the platelet membrane is responsible for the shape change.

The primary wave may require a lower cytosol calcium concentration than the secondary wave ---- the induction of the release reaction, and may involve a different pool of calcium ions (Malagodi & Chiou, 1974; Charo, Feinman & Detwiler, 1976; Le Breton & Dinerstein, 1977).

The secondary wave is calcium-dependent (Feinman & Detwiler, 1974). Since the dependency on external calcium is variable,

the internal calcium stores are thought to be mobilized for the release reaction. The dense tubular system (White, 1972; Kaser-Glanzmann, Jakabova, George & Luscher, 1978) (analogous to the sarcoplasmic reticulum of muscle), mitochondria and alpha-granules have been postulated to be the source of calcium released at this stage. The external calcium may play a role as trigger calcium to release calcium from the intracellular calcium stores.

Recently, it has been shown that the diacylglycerol compound 1-oleoyl-2-acetyl-glycerol (OAG), 12-O-tetradecanoyl phorbol -13-acetate (TPA), collagen and thrombin evoke aggregation and release reaction without elevation of intracellular free calcium above the basal level of Ø.1 μM, suggesting the existence of alternative triggers for platelet secretory exocytosis (Rink, Smith & Tsien, 1982).

Platelets possess prostaglandin synthetase activity. Needleman, Moncada, Bunting, Vane, Hamberg and Samuelsson (1976) have shown the existence of TxA2 synthetase in both human and horse platelet microsomes. Raz and Aharony (1978) have demonstrated PGH2-to-PGE2 isomerase activity in platelets treated with imidazole to remove TxA2 synthetase activity. PGD2 is also synthesized and released in sufficient quantity to affect platelet function (Oelz, Oelz, Knapp, Sweetman & Oates. 1977). And it has been found that serum albumin has PGH2-to-PGD2 isomerising activity (Hamberg & Fredholm, 1976).

It has been shown that PGI2 is synthesized in the cells of the blood vessel wall (Moncada, Gryglewski, Bunting & Vane,

1976a, 1976b); platelets lack the enzyme PGI2 synthetase.

The prostaglandins and their analogues exert powerful effects on platelets. PGI2, PGE1 and PGD2 inhibit the aggregation of platelets in vitro. The mechanism is thought to be activation of adenyl cyclase. leading to increased cyclic AMP levels. PGI2 is 30-50 times more potent than PGE1 for this effect. PGE2 exerts variable effects on platelets: it is a potentiator of the secondary aggregation wave at low concentrations (below 1 μM) and an inhibitor at higher concentrations. TxA2 is a very powerful inducer of platelet aggregation and the platelet release reaction.

It has been suggested that platelets possess PGI2 and TxA2 receptors (Whittle, Moncada & Vane, 1978; Needleman, Minkes & Raz, 1976; Needleman, Raz, Minkes, Ferrendelli & Sprecher, 1979). The existence of TxA2 receptors is further confirmed by blockade of the effect of TxA2-like agonists by a specific TxA2 receptor antagonist EP 045 (Jones, Wilson, Armstrong, Peesapati & Smith, 1983). PGE1 is thought to interact with the PGI_2— receptor. (Whittle, Moncada & Vane, 1978; Miller & Gorman, 1979).

There is some evidence suggesting that PGD2 has its own receptor, distinct from that of PGI2, on platelets. PGE1 and PGI2 inhibit the aggregation of platelets of all mammalian species studied whereas PGD2 is inactive in the rat, and relatively inactive in the rabbit and cat (Smith, Ingerman, Kocsis & Silver, 1974; Jones, Wilson & Marr, 1979). Miller and Gorman (1979) showed that there was no cross-desensitazation between PGI2/PGE1 and PGD2. N-0164,

sodium p-benzyl-4- (1-oxo -2- (4-chlorobenzyl) -3 phenylpropyl) phenyl phosphonate, abolishes the inhibition of platelet aggregation caused by PGD2, but had no effect on the inhibition caused by PGE1 and PGI2. Binding studies with prostanoid radio-ligands give further support to this hypothesis. The ability of PGs to displace bound (3H)-PGI2 from the high-affinity site is PGI2>> PGE1>> PGF1a, 6-keto PGF1a> PGD2, PGE2, PGF2a (Siegl, Smith, Silver, Nicolaou & Ahern, 1979); for (3H)-PGD2 binding, the potency is PGD2>> PGE1, PGI2, PGE2, TxB2 and PGF2a (Siegl, Smith & Silver, 1979, 1980). Schafer, Cooper, O'Hara and Handin (1979), and Cooper and Ahern (1979) showed essentially identical results with those of Siegl et al., except for the fact that PGI2 was a more potent competitor for (3H)-PGE1 binding than PGE1. Those species whose platelets are sensitive to PGD2 are those whose plasma is most active in converting endoperoxides into PGD2 (Whittle, Moncada & Vane, 1978).

It has been suggested that human platelets have a different type of TxA2 receptor than smooth muscle does (Needleman, Minkes & Raz, 1976; Needleman, Raz, Minkes, Ferrendelli & Sprecher, 1979; MacIntyre, Salzman & Gordon, 1978; Fitzpatrick, Bundy, Gorman & Honohan, 1978; Ohuchida, Hamanaka & Hayashi, 1981). Since few TxA2 analogues have been tested on rat platelets, we have used them to characterise the "thromboxane" receptor present. The ability of thromboxane receptor antagonists to block these effects was also studied. We have found that PGE2 potentiates the aggregatory action of EP 011 and 11.9-epoxymethano PGH2; for

comparison, human platelets were also studied.

METHODS

Measurement of Platelet Aggregation

Human blood was withdrawn from the antecubital vein of healthy volunteers into ACD anticoagulant (10 ml/50 ml blood) and centrifuged at 200 x g for 20 min. ACD contains disodium hydrogen citrate (1.5 H_2O) 2.0 g and glucose 3.0 g per 100 ml distilled water. Platelet-rich-plasma (PRP) was collected. Rat blood were collected in ACD (1ml/9 ml blood) with heparin 10 u/ml from cannulated abdominal aorta during ether anaesthesia and centrifuged at 200 x g for 20 min.

Rat platelets were left at room temperature for 2-3 hours to allow them to stabilize. Platelet aggregation was monitored optically using an aggregometer manufactured by H. Upchurch & Co. Ltd. and changes in light transmission were recorded on a potentiometer recorder (Servoscribe 1s).

Citrated PRP was added to Krebs solution and 0.9% NaCl solution in a 2.5 ml glass cuvette to make a final volume of 1 ml. The formulae are in Table P.1.

Cuvettes and iron rods (for stirring) were siliconized with 2% dichloro- dimethylsilane in toluene and washed twice with methanol. After incubation for 2 min (unless otherwise stated) at 37°C with constant stirring (1000 rev/min), the aggregating agent was added in 0.1 ml 0.9% NaCl solution. Inhibitory or enhancing drugs in 0.1 ml 0.9%NaCl solution were added 2 min prior to the addition of the aggregating

Table P.1 The recipes for testing platelet aggregation

	Amount of PRP	Krebs	Ø.9% NaCl	Agent
Human (ml)	Ø.5	Ø.3	Ø.1	Ø.1
Rat (ml)	Ø.3	Ø.4	Ø.2	Ø.1

agent (replacing 0.1 ml 0.9% NaCl solution).

Measurement of Platelet Secretion

Rat PRP was pre-incubated at 37°C for 30 min with 1 µg/ml (final concentration) 5-hydroxy-(side chain)2-[14C]-tryptamine which is incorporated into platelet dense bodies. PRP (0.3 ml) was diluted with 0.4 ml Krebs solution and 0.2 ml 0.9% NaCl solution. 2 Min after the addition of an agonist to the stirred platelet samples, 200 µl samples were withdrawn into 1.5 ml Eppendorf tubes. Release was terminated by the addition of 0.8 ml ice-cold 0.4% w/v EDTA in 0.9% NaCl and immediate centrifugation (15000 x g, 20°C, 30 s) in an Eppendorf centrifuge. Subsamples (0.5 ml) of cell-free supernatant were transfered into scintillation vials containing 10 ml of scintillant (2 parts PCS* and 1 part toluene). Radioactivity was counted in a Philips PW 4540 liquid scintillation counter. It gave a value of counts per minute (CPM) for each sample. The increase in supernatant radioactivity above control was calculated and expressed as a percentage of the radioactivity released by the highest concentration of the aggregating agent.

* Amersham Corp.

RESULTS

Rat Platelets

On rat platelets at concentrations from 0.01-1.0 μM, ADP elicited a typical pattern of platelet aggregation: a shape change at the lowest effective concentration (0.01-0.05 μM), a modest reversible clumping of platelets ("primary aggregation") at higher concentrations (0.05-0.2 μM), and a more intense irreversible aggregation at 0.2 μM and above at which the primary and secondary aggregation response merged (Figure P.1). It was often difficult to find out the narrow range of intermediate concentrations where primary and secondary responses are kinetically distinct, resulting in biphasic aggregation patterns.

EP 011, the most active agent among all prostanoids that induced platelet aggregation, gave a different pattern of response from that of ADP: reversible aggregation was difficult to produce and the transition into a secondary wave was marked (Figure P.2). EP 011 induces a shape change in rat platelets at concentrations of 20-100 ng/ml. the biphasic aggregation at 100-200 ng/ml and the merged aggregation response at higher concentrations. 11,9-Epoxymethano PGH2, 9,11-azo PGH2, 15-oxo EP 011, CTA2, PTA2 and 16,16-dimethyl PGF2a were also tested. Responses were determined in the following way. Adding 0.1 ml 0.9% NaCl increased light transmission to a new level, which was

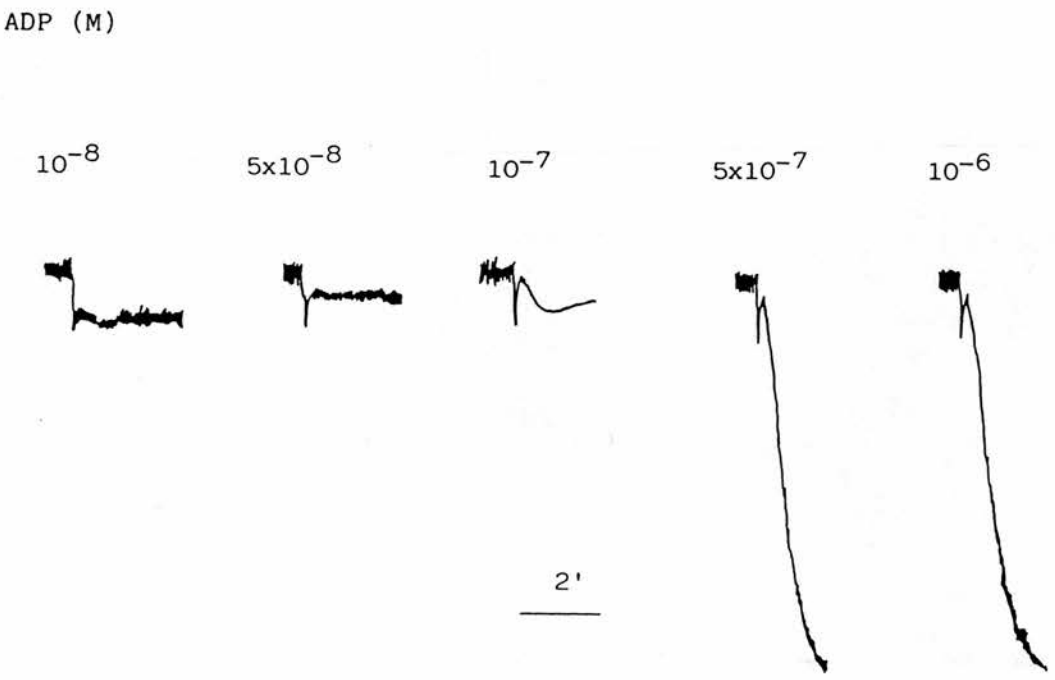


Figure P.1 Aggregation of rat platelets in vitro:

ADP produces a shape change and a reversible aggregation at low concentrations and irreversible aggregation at higher concentrations.

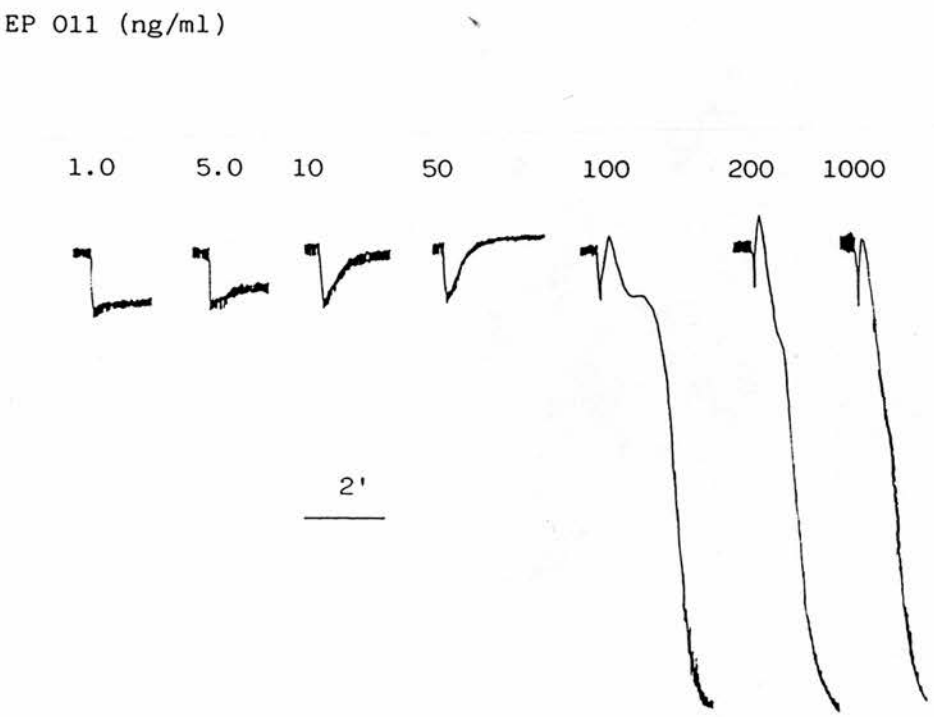


Figure P.2 Aggregation of rat platelets in vitro:
EP 011 produces a shape change at low concentrations and irreversible aggregation at higher concentrations. At 100 ng/ml EP 011 induces a biphasic aggregation.

taken as a zero level; the decrease in light transmission due to platelet shape change produced by a prostanoid after a 2 min period was measured and expressed as a percentage of the maximum shape-change response. For aggregation, the response was taken as the clear increase in light transmission above the basal level 2 min after adding the agent. Log concentration-response curves for shape change and aggregation were plotted.

The threshold concentrations of each prostanoid for shape change and aggregation were determined and the results of these studies are shown in Table P.2. 15-oxo EP Ø11, CTA2 and PTA2 produced shape change only. The relative potencies of prostanoids were measured, EP Ø11 was taken as a standard (for CTA2, 11,9-epoxymethano PGH2 was taken as a standard). The results are summarized in Table P.3. If a full agonist such as EP Ø11 is added 2-5 min after treatment with 15-oxo EP Ø11, PTA2 or CTA2 at 1 μg/ml, its aggregating activity is inhibited (Figure P.3). When a concentration of EP Ø11 which produced a shape change similar in extent to that of 15-oxo EP Ø11, PTA2 or CTA2 was used, the action of a further dose of EP Ø11 was not opposed.

Effects of TxA2 Receptor Antagonists

EP Ø45, EP Ø92 and EP 116 blocked responses to EP Ø11, 15-oxo EP Ø11, and PTA2. The irreversible aggregatory action of arachidonic acid (75 μg/ml) was also blocked. Log concentration-response curves for a full agonist or a partial agonist were constructed in the presence of fixed concentration of an antagonist. EP 116 vs. EP Ø11, EP116 vs.

Table P. 2 Threshold concentrations of prostanoids for rat platelet aggregation and shape change.

Compound	Threshold concentration	
	shape change (ng/ml)	aggregation (ng/ml)
(±) EP 011	0.80 1.0 1.0 1.1 1.2 1.4 1.5 (1.1±0.092, n=7)	100 100 100 100 100 100 200 (110±14, n=7)
11,9-epoxymethano PGH_2	2.0 3.0	1000 5000 5000
9,11-azo PGH_2	2.5 6.0 8.0	1000 1000
(±) 15-oxo EP 011	70 100 130 150 (110±18, n=4)	-
CTA_2	50	-
PTA_2	20 50 70 100 120 (72±18, n=5)	-
16,16-dimethyl $PGF_{2\alpha}$	1000	-

Threshold concentrations of a prostanoid indicate the concentrations which produced 5% of its own maximum response.

Values in parentheses indicate mean±s.e..

- denotes that no aggregation occurred at concentration up to 10 µg/ml for CTA_2 and PTA_2, and up to 5 µg/ml for 15-oxo EP 011.

Table P. 3 Relative activities of prostanoids on rat platelet in vitro

Compound	Equipotent molar ratios (EP 011=1.0)	
	shape change	aggregation
9,11-azo PGH_2	3.1 5.7 11	5.6
11,9-epoxymethano PGH_2	6.3 9.1 13 29 29 (17±4.9, n=5)	27 33
(±) 15-oxo EP 011	71 120 140 170 200 320 (170±35, n=6)	
PTA_2	110 170 200 340 (210±49, n=4)	

	Equipotent molar ratios (11,9-epoxymethano PGH_2=1.0)
	shape change
CTA_2	9.6

The standard agonist EP 011 was racemic. For CTA_2 11,9-epoxymethano PGH_2 was used as a standard agonist.

Values in parentheses indicate mean±s.e..

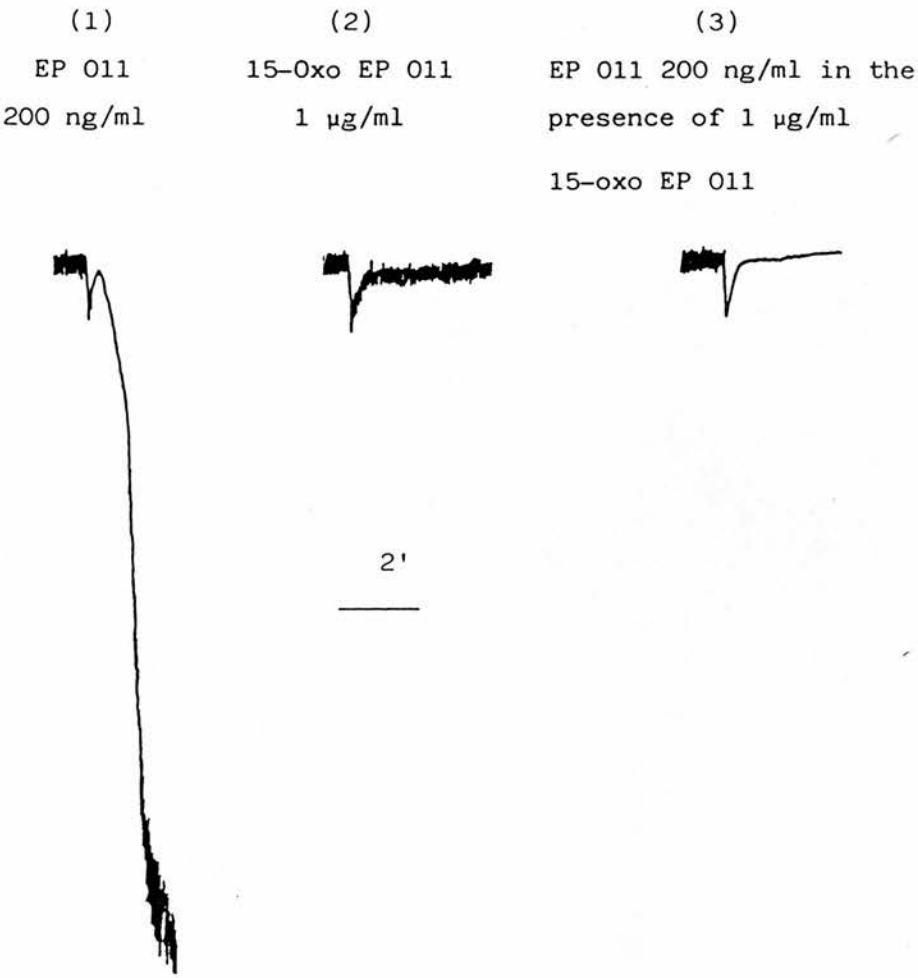


Figure P.3 Rat platelets: interaction of 15-oxo EP 011 with EP 011. 15-Oxo EP 011 produces a shape change only and opposes the action of EP 011. In trace 3, EP 011 was added 5 min after 15-oxo EP 011 treatment.

15-oxo EP Ø11 and EP Ø45 vs. PTA2 were studied. With all of the analogues examined approximately parallel shifts to the right of the agonist concentration-response curve were produced. Dose-ratios are shown in Table P.4. From the Gaddum-Schild equation affinity constants were obtained: $9.4x10^{6}$ M^{-1} (shape change), $8.0x10^{6}$ M^{-1} (aggregation) for EP 116 vs. EP Ø11; $7.8x10^{6}$ M^{-1}, $9.8x10^{6}$ M^{-1} and $5.8x10^{6}$ M^{-1} (shape change) for EP 116 vs. 15-oxo EP Ø11 at 1ØØ ng/ml, 2ØØ ng/ml and 5ØØ ng/ml EP 116, respectively; $2.1x10^{6}$ M^{-1} (shape change) for EP Ø45 vs. PTA2.

Effects of PGI2, PGE1 and PGD2.

11,9-Epoxymethano PGH2 was used as a shape change inducer. PGI2 at 5 ng/ml or PGE1 at 2Ø ng/ml inhibited 1ØØ ng/ml 11,9-epoxymethano PGH2-induced shape change. PGD2 at concentrations up to 1 µg/ml produced no effect.

Effects of PGE2 Analogues.

PGE2 at concentrations up to 2 µg/ml produced neither shape change nor aggregation responses, but it enhanced the aggregatory action of EP Ø11, 11,9-epoxymethano PGH2 and 9,11-azo PGH2. Using EP Ø11 (5Ø ng/ml) as a shape change agent, the threshold concentration for PGE2 to convert the shape change into an irreversible aggregation response was found to be 5Ø ng/ml. Increasing the concentration of PGE2 did not produce greater enhancement. Table P.5 lists the combination of the concentration of the thromboxane mimetic which gives a near maximal shape change together with the minimal concentration of the PGE analogue which will convert

Table P. 4 Effects of TxA_2 antagonists on shape change and aggregation response to some TxA_2 analogues on the rat platelets.

Treatment compound	dose (ng/ml)	Agonist	Dose-ratios for shape change	aggregation
EP 116	500	EP 011	12	*100
EP 116	100	15-oxo EP 011	2.8	
EP 116	200	15-oxo EP 011	5.4	
EP 116	500	15-oxo EP 011	7.6	
EP 045	5000	PTA_2	2.8	

EP 011 and 15-oxo EP 011 were racemic.

* indicates the full dose-response curve for EP 011 in the presence of EP 116 was not established; the dose-ratio was quantified related to the response at 5% maximum response level of EP 011 in the absence of EP 116.

Table P. 5 Combinations of minimal concentrations of PGE_2 analogues and threshold concentrations of TxA2 analogues which transformed shape change into aggregation response.

	PGE_2	16,16-dimethyl PGE_2		ICI 80205
	50 ng/ml	10 ng/ml	20 ng/ml	20 ng/ml
11,9-epoxymethano PGH_2	100 ng/ml		100 ng/ml	
EP 011	50 ng/ml	50 ng/ml		50 ng/ml
9,11-azo PGH_2	500 ng/ml			

EP 011 and ICI 80205 were racemic. Except for 9,11-azo PGH_2 the experiments were conducted on the same batch of rat platelets.

their shape change response into an irreversible aggregation wave. 16,16-dimethyl PGE2 was more potent than PGE2, but similar to ICI 80205. Typical full dose-response curves for shape change and aggregation responses induced by EP 011 in the presence and absence of a fixed concentration of PGE2 are shown in Figure P.4. Below the threshold concentration of EP 011 mentioned above, PGE2 had little effect on the shape change induced by EP 011, but above that concentration PGE2 transformed shape change into aggregation or potentiated aggregative responses. PGE2 had little effect on both shape change and aggregation produced by ADP.

Effects of PGE2 on release of [14C]-5-HT.

In order to investigate the mechanism behind the enhancement by PGE2 of EP 011 induced aggregation a set of experiments was performed on release of [14C]-5-HT. The results are shown in Figure P.5. With EP 011 alone shape change was not associated with secretion of 5-HT, but resulted in uptake of the labelled 5-HT; the irreversible aggregation wave was accompanied by secretion of [14C]-5-HT in rat platelets. In the presence of 50 ng/ml PGE2, there was a marked increase in secretion of 5-HT during aggregation induced by EP 011. PGE2 also potentiated secretion of [14C]-5-HT induced by 11,9-epoxymethano PGH2.

Effects of Verapamil and Calcium.

Verapamil, a calcium channel blocker, was tested on rat platelets against EP 011. Verapamil at 10-100 μM partially inhibited irreversible platelet aggregation induced by

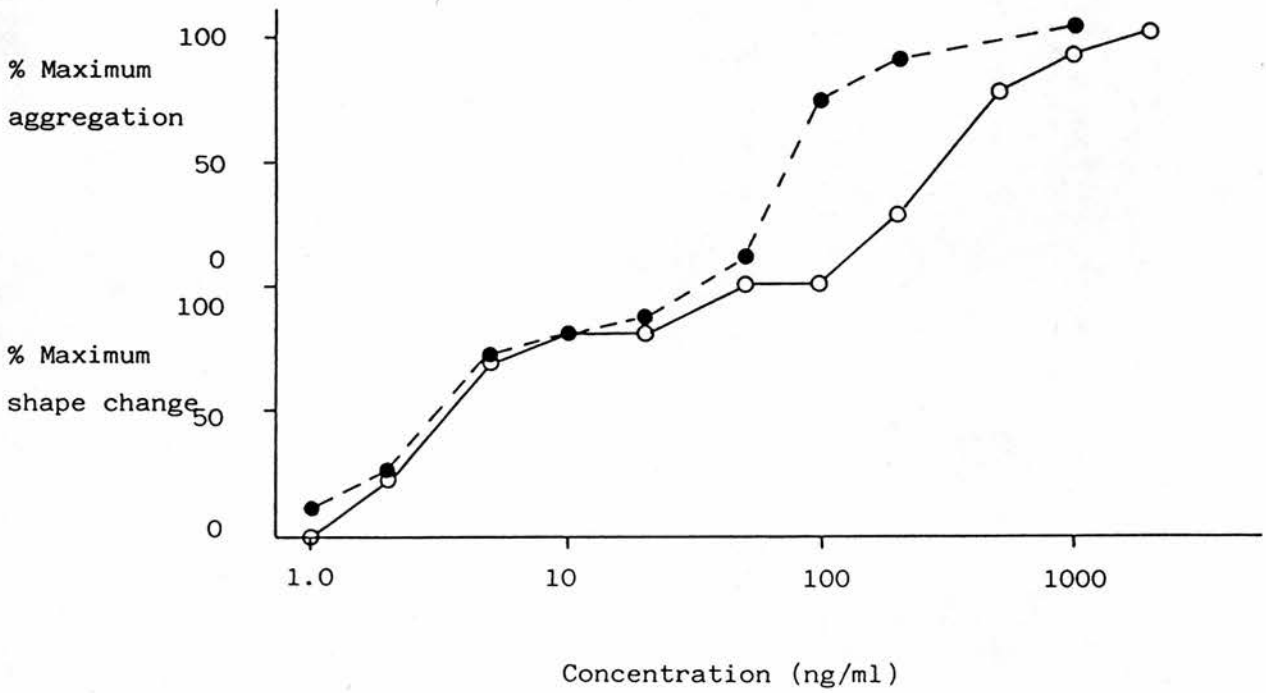


Figure P.4 Rat platelets: potentiating effects of PGE_2 on the aggregating response to EP 011. The figure shows the dose-response curve for EP 011 acting alone (open circle) and the corresponding curve (solid circle) in the presence of 50 ng/ml PGE_2. PGE_2 was added 2 min before adding EP 011.

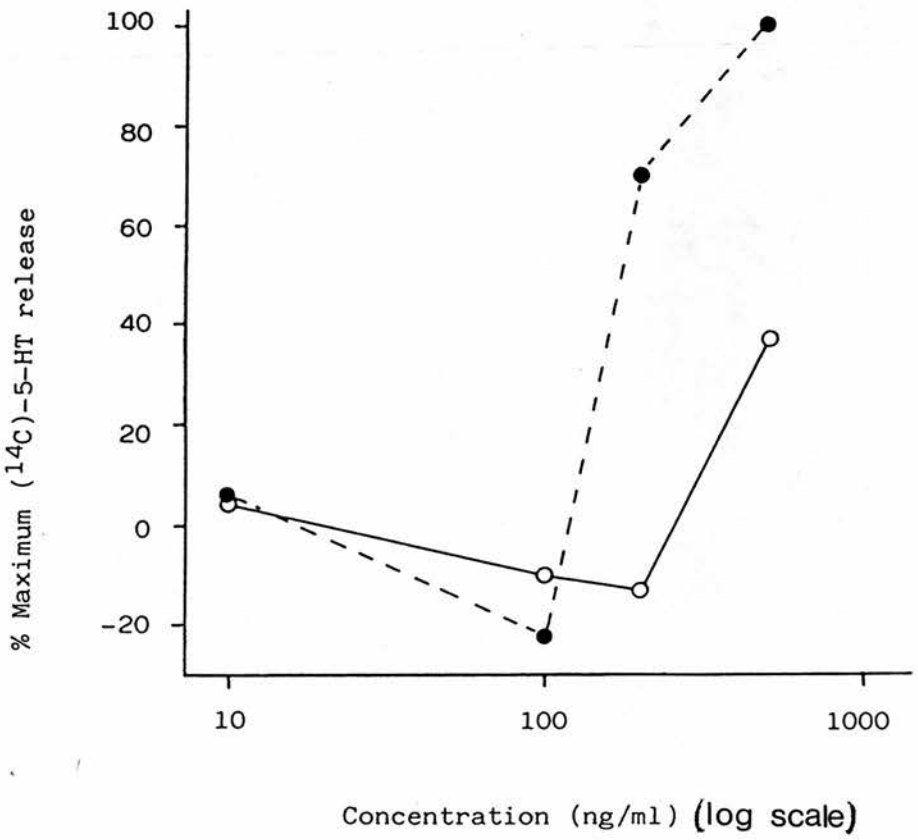


Figure P.5 Rat platelets: potentiation by PGE_2 of the release of (^{14}C)-5-HT induced by EP 011. The open circle indicates EP 011 acting alone and the solid circle indicates EP 011 acting in the presence of 50 ng/ml PGE_2. PGE_2 was added a min before adding EP 011.

EP Øll (Figure P.6).

Increasing the calcium concentration by 1, 2 and 1Ø mM above that present in the citrated PRP - Krebs mixture did not affect the shape change or aggregation response to EP Øll. Higher concentration (>1Ø mM) of calcium chloride itself produced platelet shape change.

Effects of Indomethacin

Rat platelets were preincubated with indomethacin for 25-35 min before adding an aggregating agent. Indomethacin at 1ØØ μM inhibited the aggregation induced by arachidonic acid(AA). The results are shown in Figure P.7. Effects of indomethacin on the aggregating response to EP Øll were inconsistent. In one set of experiments, indomethacin at 1ØØ uM inhibited aggregation produced by EP Øll, transforming irreversible aggregation into reversible aggregation; in another set of experiments indomethacin at 1ØØ μM had no influence on EP Øll induced aggregation.

Human Platelets

Human PRP was freshly prepared. The sensitivity to TxA2 analogues was markedly decreased when platelets was left overnight. Doses of 11,9-epoxymethano PGH2 tested were 1ØØ, 2ØØ, 3ØØ and 5ØØ ng/ml. Reversible aggregation was seen with 1ØØ and 2ØØ ng/ml, and irreversible aggregation with 3ØØ ng/ml and above. PGE2 inhibited the aggregation induced by 11.9-epoxymethano PGH2 (Figure P.8).

EP 011
1 µg/ml

EP 011 1 µg/ml in the presence of 10^{-4} M verapamil

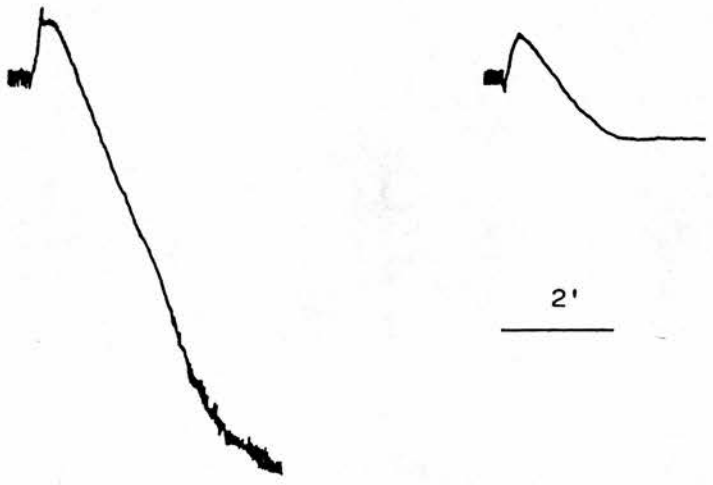


Figure P.6 Rat platelets: inhibitory effect of verapamil on the aggregating response to EP 011. The figure shows EP 011 acting alone (left), and in the presence of verapamil 10^{-4} M (right). The platelets were pre-incubated with verapamil for 5 min before adding EP 011 in the experiment shown on the right panel.

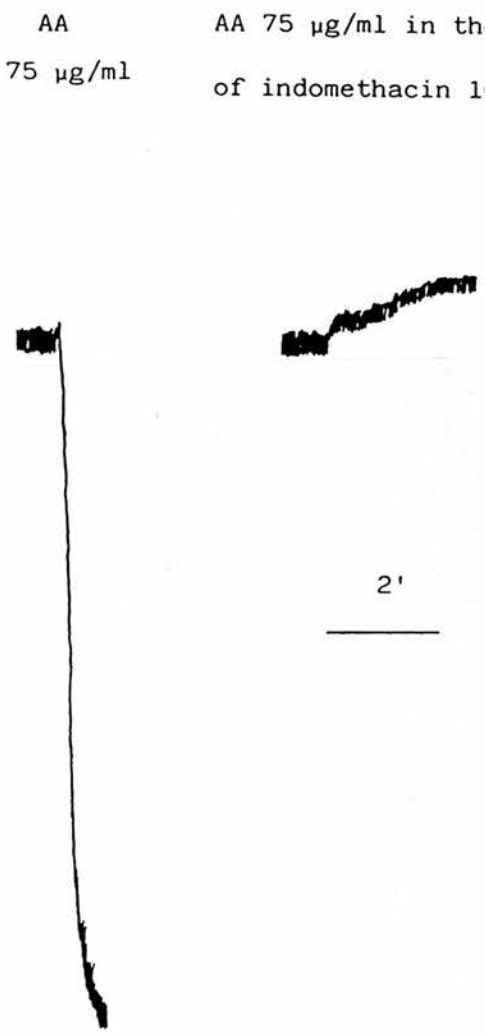


Figure P.7 Rat platelets: inhibitory effects of indomethacin on the aggregating response to AA. The figure shows AA 75 µg/ml acting alone (left) and in the presence of indomethacin (right). The platelets were pre-incubated with indomethacin 10^{-4} M for 30 min before adding AA.

AA: Arachidonic acid

11,9-Epoxymethano PGH_2
300 ng/ml

11,9-Epoxymethano PGH_2 300 ng/ml
in the presence of 100 ng/ml PGE_2

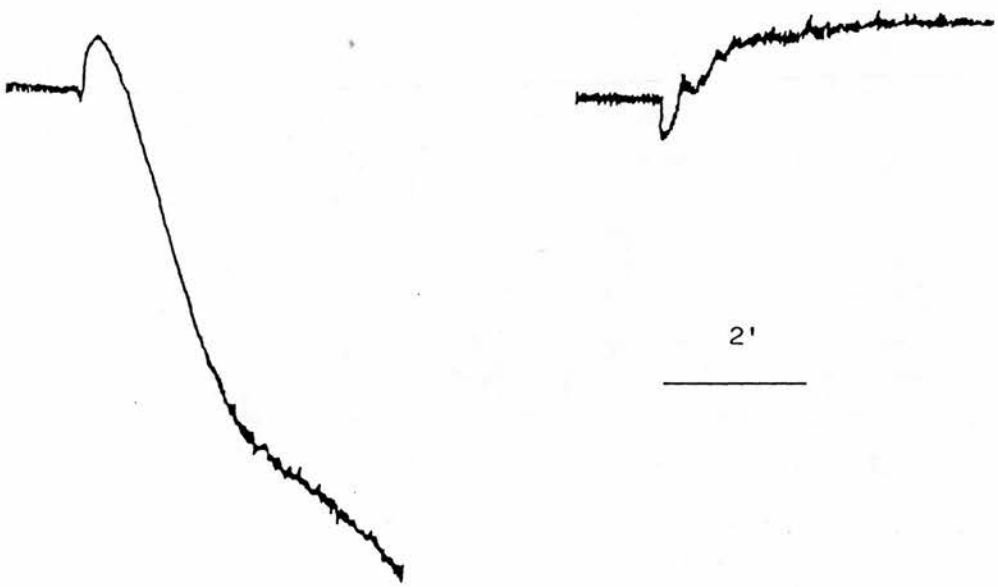


Figure P.8 Human platelets: inhibitory effects of PGE_2 on the aggregating response to 11,9-epoxymethano PGH_2. The left panel shows 11,9-epoxymethano PGH_2 300 ng/ml acting alone. The right panel shows 11,9-epoxymethano PGH_2 300 ng/ml acting in the presence of PGE_2 100 ng/ml; the platelets were pre-incubated with PGE_2 for 2 min before adding 11,9-epoxymethano PGH_2.

However, in another group of experiments PGE2 and 16,16-dimethyl PGE2 at 100 ng/ml potentiated the aggregation response elicited by 11,9-epoxymethano PGH2 and EP 011 (Figure P.9). EP 011 was about 5 times more potent than 11,9-epoxymethano PGH2 in inducing aggregation in human platelets. PTA2 at 10 µg/ml did not show any response, but opposed EP 011-induced aggregation. 15-Oxo EP 011 at 5 µg/ml was inactive and had no effect on the response to EP 011.

11,9-Epoxymethano PGH_2 500 ng/ml

11,9-epoxymethano PGH_2 500 ng/ml in the presence of PGE_2 100 ng/ml

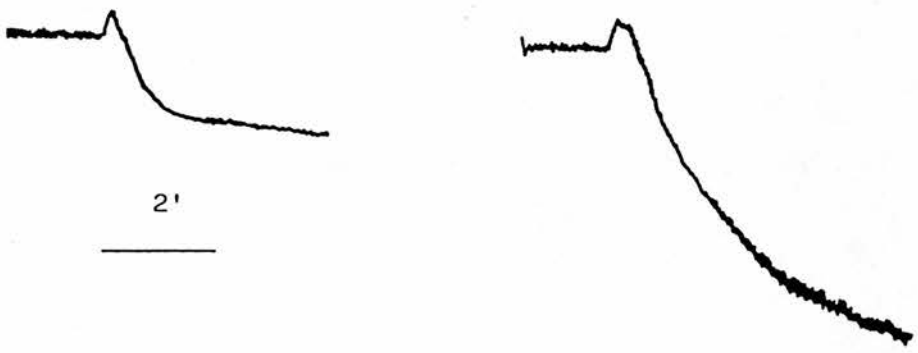


Figure P.9 Human platelets: potentiating effects of PGE_2 on the aggregating response to 11,9-epoxymethano PGH_2. The left panel shows 11,9-epoxymethano PGH_2 500 ng/ml acting alone. The right panel shows 11,9-epoxymethano PGH_2 acting in the presence of PGE_2 100 ng/ml; the platelets were pre-incubated with PGE_2 for 2 min before adding 11,9-epoxymethano PGH_2.

DISCUSSION

There are several lines of evidence suggesting that the TxA2 receptor of human platelets is different from that of smooth muscle:

(1) Both TxA2 and TxA3 are vasoconstrictor (TxA2> TxA3) whereas only TxA2 induces platelet aggregation and TxA3 inhibits aggregation (Needleman, Minkes & Raz, 1976; Needleman, Raz, Minkes, Ferrendelli & Sprecher, 1979).

(2) 15-Oxo EP Ø11 has no effect on human platelets, but it is a full agonist on smooth muscle (Jones, R.L., Personal communication).

(3) CTA2 is a full agonist on smooth muscle yet only causes shape change and antagonises human platelet aggregation induced by AA or 11,9-epoxymethano PGH2 (Jones, R.L., Personal communication).

(4) The sodium salt of rac- 9,11:11,12- dideoxa- 9,11:11,12- diepithio TxA2 (both ring oxygens in TxA2 are replaced by sulphur atoms) contracts the rat aortic preparation but has no effect on human platelet aggregation (Ohuchida, Hamanaka & Hayashi, 1981).

(5) 9,11-epoxyiminoprosta- 5,13- dienoic acid (9,11-EIP) is a TxA2 antagonist in human platelets, but has intrinsic agonist properties for smooth muscle, contracting the rat aorta strip (Fitzpatrick, Bundy, Gorman & Honohan, 1978).

Our results suggest that the TxA2 receptor in rat platelets differs both from that in smooth muscle and from that in human platelets. It seems to represent a new type of TxA2

receptor.

The differences between rat platelets and smooth muscle are evident: CTA2 and 15-oxo EP Ø11, which are full agonists on the smooth muscle preparations we have used, show partial agonist activity on rat platelets.

In the rat platelets, CTA2 caused shape change only and opposed the effect of EP Ø11, indicating partial agonist properties. In this aspect, rat platelets resemble human platelets. However, 11,9-epoxymethano PGH2 and 9,11-azo PGH2 are much weaker relative to EP Ø11 in rat platelets than in human. Considering that EP Ø11 might trigger the synthesis and release of endogenous PGE2 or TxA2, indomethacin was used. The results are inconsistent. Thus we can not draw any conclusions at the present stage. As we have suggested previously EP Ø11 may have PGE2-like activity. However, since the minimal concentration for PGE2 to potentiate EP Ø11 action is 5Ø ng/ml, EP Ø11 at the concentrations we used is unlikely to have enough PGE2-like activity to enhance its own TxA2-like activity. Furthermore, 15-oxo EP Ø11, which had no effect on human platelets and was a full agonist on smooth muscle, showed partial agonist activity on rat platelets. The ability of TxA2 receptor antagonists to block both shape change and aggregation induced by EP Ø11 and 15-oxo EP Ø11 indicates that their effects are receptor-mediated.

As we have shown in the Addendum (see Figure Add.5), rat platelets can convert AA into TxB2, a TxA2 metabolite, which is confirmed by the work of Chang and Tai (1983). This

appears to imply that the receptor on which EP Ø11 and 15-oxo EP Ø11 act is a type of TxA2 receptor.

It has been suggested that there are distinct "endoperoxide" and "thromboxane" receptors. MacIntyre and Gordon (1977), and Smith, Ingerman and Silver (1977) proposed that exogenous PGG2 and PGH2 stimulate platelets directly by interacting with their own receptor since 11,9-epoxymethano PGH2 and 9,11-azo PGH2, which they assumed were stable PGH2 analogues, aggregated platelets, and PGG2 and PGH2 induced platelet aggregation in PRP without significant production of TxA2. Furthermore, in 1978 MacIntyre and Willis found that contractions of rabbit aorta induced by PGH2, but not TxA2, were suppressed by trimethoquinol at 1Ø µg/ml; pre-incubation of platelet microsomes with trimethoquinol (1Ø-1ØØ µg/ml) for up to 4 min (at Ø°C) did not suppress production of TxA2-like activity from PGH_2. Therefore, they suggested that there were distinct endoperoxide and TxA2 receptors in vascular tissue, and that the former is more susceptible to inhibition by trimethoquinol. More recently, Lampugnani and de Gaetano (1983) have reported that whilst aspirin induced a parallel suppression of shape change and TxB2 generation from AA, dazoxiben, a selective TxA2 synthetase inhibitor, completely prevented TxB2 formation but did not modify shape change. They have suggested that PG endoperoxides are effective mediators of shape change. However, these suggestions are not convincing since it has been found 11,9-epoxymethano PGH2 is a selective TxA2 mimetic (Coleman, Humphrey, Kennedy, Levy & Lumley, 1981), and PGG2 and PGH2 can be active agonists on

the TxA2 receptor since they are structurally similar to TxA2. It is going to be a very difficult task to differentiate these two receptors because of the evanescent nature of PGH_2 and TxA_2. Thus, it is uncertain whether the receptor in rat platelets is TxA2 or PGH2 receptor. Anyhow, it is a different type of receptor from that of smooth muscle or human platelets.

It was thought that the weak activity of 11,9-epoxymethano PGH2 on rat PRP was due to insufficient calcium in the milieu, but increase in calcium concentration made no improvement. Inspired by the findings that PGE2 potentiates human platelet aggregation induced by a TxA2-like substance (Weiss, Willis, Kuhn & Brand, 1976), we studied the interactions of PGE2 analogues with TxA2 analogues. We have found that PGE2, 16,16-dimethyl PGE2 and ICI 80205 potentiate aggregation of rat platelets induced by TxA2 analogues. As shown in the results, PGE2 facilitates the release of 5-HT by EP 011. It is believed that the aggregation and release reaction require extracellular calcium, and we have shown that verapamil, a calcium channel blocker, inhibits aggregation induced by EP 011. Thus, PGE2 may produce the potentiation by helping EP 011 manoeuvre extracellular calcium. Alternatively, PGE2 may have an intracellular acting point to facilitate the release of calcium from intracellular stores by trigger calcium which enters the cell due to activation of TxA2 receptor by EP 011. Thus, in rat both TxA2 and PGE2 are important in platelet aggregation.

In contrast to the work by Ashida and Abiko (1979), who reported that PGE2 enhanced the aggregating action of ADP, we have found PGE2 has little effect on the response to ADP. Aspirin inhibits aggregation induced by ADP (Tuong, Ferrand, Aubert, Loubrie & Tuong, 1982), which indicates ADP can stimulate endogenous synthesis of prostanoids, probably TxA2. In our study, the platelets were left for 2-3 h for stabilization, which might attenuate the synthesis of TxA2 stimulated by ADP. If this is true, the PGE2 potentiating effect on ADP-induced aggregation in the study of Ashida and Abiko may be produced through endogenously synthesized TxA2.

As we mentioned in the introduction, platelet shape change is caused by release of membrane or receptor-bound calcium. PGE2 does not affect the shape change induced by low concentrations of EP Ø11. During shape change the increased uptake of 5-HT is possibly due to the increase in platelet size, and PGE2 had little effect on the 5-HT movement. This seems to indicate that PGE2 does not enhance the release of membrane or receptor-bound calcium induced by activation of TxA2 receptor. It is known PGI2, PGEl and PGD2 inhibit platelet aggregation by increasing the cyclic AMP level in platelets, and cyclic AMP acts as a calcium antagonist by enhancing calcium uptake into the platelet calcium storage sites in the dense tubular system (Kaser-Glanzmann, Gerber & Luscher, 1979), or cyclic AMP prevents membrane or receptor-bound calcium release. This may explain the PGI2/PGEl inhibition of rat platelet shape change. PGD2 failed to produce the inhibition since rat platelets lack PGD2 receptors.

The cold-induced shape change seems to be mediated via a different mechanism since PGE1 can not prevent it (Zucker & Peerschke, 1980).

In human platelets PGE2 produced varied results: it may inhibit or enhance aggregation elicited by TxA2 analogues. It is possible that the PGE2 receptor mediates both potentiation and inhibition, and the outcome depends on the dominant effect which varies with donors.

ADDENDUM

Metabolism of AA* to TxA2 by Rat Platelets and Bullock Iris Sphincter

Basic Principles of GC/MS.

GC/MS consists of two parts: gas chromatography and mass spectrometry. The gas chromatography column is a tube containing the solid (support) on which the liquid phase is coated. A mixture of two or more components is vapourised into the column through which an inert gas (carrier) is flowing. Because of the relative retention characteristics, partitioning of the mixture between the gas and liquid phases results in a separation of the individual components with time. The separated components then pass into the mass spectrometer. Electron bombardment of the components in a high vacuum chamber give rise to charged particles. The ions are accelerated by a potential (V) and made to move in a curved trajectory by electric and magnetic fields. The radius of curvature $(R)_{\lambda}$ of these ions is determined by their mass-to-charge (m/e) ratio and the strength of magnetic field (H) such that $m/e = H^2R^2/2V$ and only ions of a particular m/e will be focussed at the collector (detector) for particular values of H and V.

In gas chromatography, carrier gas flow rates of 30 ml/min are normally used. If the carrier gas, as well as the individual components of the mixture, were put into mass spectrometer, the ionisation process would become very

*AA = arachidonic acid

inefficient due to collisional effects and sensitivity would suffer. Therefore, a major part (90%) of the carrier gas (usually Helium) has to be removed selectively prior to entry into the source. This is achieved by the use of a molecular separator.

Identification of a compound relies on the detection of one or more representative ions appearing at the appropriate retention times.

The quantification of prostanoids by GC/MS can be most accurately conducted by using stable isotopes as internal standards. For this purpose, prostanoids are synthesized that contain four or more deuterium atoms in non-exchangeable positions of the molecule, for example, 3,3,4,4-tetradeutero TxB2. A defined amount of the deuterated internal standard is added to the biological sample and passes through all steps of the work-up procedure. The deuterated prostanoids behave nearly identically to the endogenous prostanoid in the biological sample on extraction, derivatization and chromatography. In the final steps of analysis, the mass spectrometer can discriminate between the endogenous prostanoid and the internal standard, the mass of which is dependent on the number of incorporated deuterium atoms (for 3,3,4,4- (2H)4-TxB2, the difference is four mass units). The ratio of protium peak height to deuterium peak height is determined over the range of a standard curve and the concentration of TxB2 in the sample is determined from the ratio of protium to deuterium in the sample.

The GC/MS data were obtained by using a Pye Unicam 204 gas chromatograph coupled to a VG micromass 70-70F mass spectrometer. The gas chromatograph was equipped with a spiral column (either 1.5 m x 4 mm or 3 m x 4 mm) packed with 3% OV1 in 100-200 mesh Supelcopart (Supelco, Inc.). The temperature of the column was fixed between 250-270°C, the separator temperature was 250°C, the ion source temperature 250 °C, the basic accelerating voltage 4 KV and the electron energy 22 or 70 eV. Helium was used as the carrier gas with a flow rate of 30 ml/min.

Derivatisation of TxB2

Samples must be derivatised to be both volatile and thermally stable. Derivatisation provides suitable ions in the mass spectrum for detection and assay by GC/MS. However, the ion must not coincide with a column bleed peak (Figure Add.1), but should be reasonably close in mass to minimise the loss in sensitivity which occurs as the voltage jumps from the column bleed peak to the ion, throughout the assay. The column bleed peak is used as a reference to provide accurate switching between monitored peaks.

Diazomethane was generated from diazald (N-methyl-N-nitroso- p- toluene- sulphonamide, Aldrich), ethanol and potassium hydroxide (KOH), and displaced with ether-vapour-saturated nitrogen into a tube containing cold diethyl ether. An appropriate amount of TxB2 (UpJohn Co., Kalamazoo) in ethylacetate was dispensed into an Eppendorf tube and blown dry. The TxB2 was taken up in a few drops of methanol

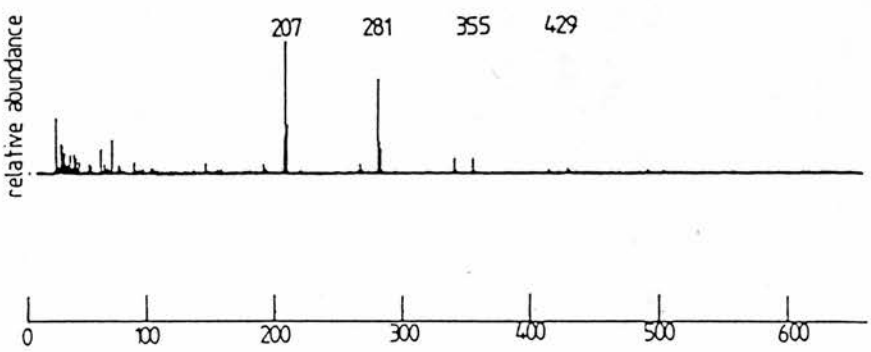


Figure Add.1 Mass spectrum of column bleed peaks.

(Rathburn Chemicals), Ø.3-Ø.4 ml ethereal of diazomethane added, and left for 5 min before being blown dry on a heating block and desiccated. Butoxyamine hydrochloride, 3-5 drops (5 mg/ml) in pyridine was then added. The Eppendorf tube with the mixture in it was sealed and kept at 6Ø°C for 9Ø min. The pyridine was blown off using a heating block and the tube desiccated in a vacuum chamber before 2Ø µl BSTFA (Sigma)(N,O-bis (trimethylsilyl) trifluoroacetamide) was added and this was sealed and incubated at 6Ø°C for 15 min. TxB2 methyl ester, butyloxime, TMS ether was injected in the BSTFA into the GC/MS and from the total ion chromatogram the retention time and a full mass spectrum of the ions formed as the molecule splits were obtained. The m/e = 3Ø1 ion is the base peak, i.e. the most abundant ion formed (Figure Add.2). The m/e =3Ø1 ion was chosen as a suitable ion to monitor since it is formed in large amounts, being the base peak, and is suitably close in mass to the 281 column bleed. Both of these factors help to increase the sensitivity of the assay.

Since deuterated TxB2 was not available, 11-deoxy PGE1 was selected as an internal standard to account for losses in the derivatization and chromatography steps. The mass spectrum of 11-deoxy PGE1 methyl ester, butyloxime, TMS ether showed the m/e =28Ø ion to be the base peak (Figure Add.3). Again this ion is close in mass to the 281 column bleed. The m/e =28Ø ion could be formed by loss of the ω-chain (-71) and the α-chain (-144) or by the loss of the butyloxime (-73) and the α-chain (-142). Since the m/e = 28Ø ion is also the base peak of 11-deoxy PGE1, methyl ester, methyloxime, TMS ether, it

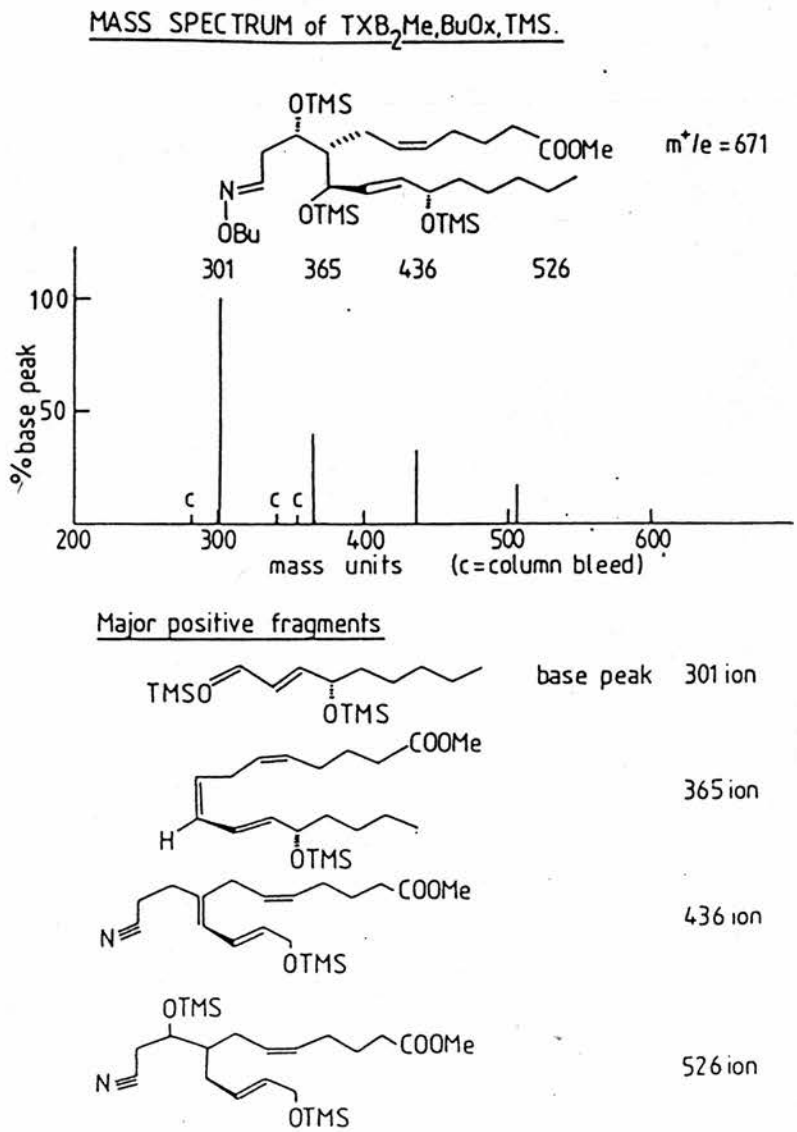


Figure Add.2 Fragmentation of TxB_2 Me, BuOx, TMS by GC-MS.

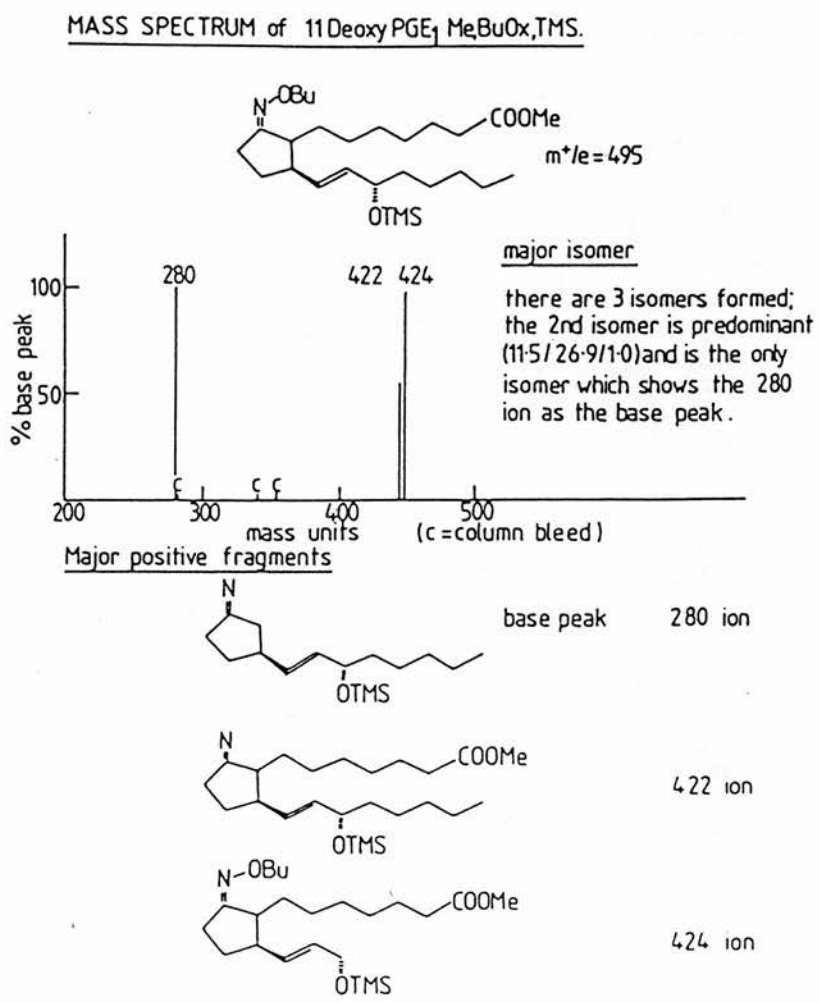


Figure Add.3 Fragmentation of 11 deoxy PGE_1 Me, BuOx, TMS by GC-MS.

must be achieved by loss of the oxime (-31) and the α-chain (-142). 11-Deoxy PGE1 methylester, butyloxime, TMS ether has a shorter retention time than the corresponding TxB2 derivative.

Multiple Ion Detection Assay

Amounts of TxB2, 0 ng, 10 ng, 20 ng, 40 ng, 60 ng and 80 ng were dispensed into Eppendorf tubes. 40 ng 11-deoxy PGE1 was added to each tube. The drugs were methylated using diazomethane, as above mentioned, and dried. The methylated drugs were oximated using butoxyamine hydrochloride at 60 °C for 90 min before being taken to dryness on a heating block and desiccated. 20 µl BSTFA were added to each tube. The tubes were kept at 60°C for 15 min and 5 µl aliquots were injected into GC column.

The program used for multiple ion detection (MID) is set up as shown in Table Add.1.

Table Add.1. GC-program for assay of TxB2 by MID

Channel	Ion	Exact mass (g)	MID voltage
1	280	280.20965	9.8000
2	281	281.05169	9.7706
3	301	301.20190	9.1170

The instrument was tuned into the 281 colunm bleed, and

electronically locked on, to facilitate its use as a reference peak. The machine was focussed on each MID voltage for 0.1 s in turn, the MID voltage regulating the accelerating voltage. The responses were recorded using a recorder (Rikadenki Mitsui Electronics (U.K.) Ltd.) while each standard was run. The ratios of peak heights of 301/280 ions were determined. Typical traces are shown in Figure Add.4 with the standard curve.

Samples to be tested were diluted to give a peak within the range of peak heights produced by 0-20 ng TxB2 and processed in the same way as the standard TxB2.

Arachidonic acid (Grade 1, Sigma), 10 mg/ml in methanol (Rathburn Chemicals), was stored in ampoules at -40°C until use.(AA)

Extraction of TxB2 from Rat PRP

Rat PRP was prepared as before and incubated in a waterbath at 37°C. 2.5 ml of rat PRP was incubated for 3 min at 37°C with 75 µg/ml palmitic acid, then 100 µg/ml AA (final concentration) was added to the PRP and allowed to act for 30 s before the reaction was quenched by the addition of 500 µl 2M HCl which gave a pH of about 3. The sample was extracted twice with ethyl acetate (Rathburn Chemicals) and the extracts taken into a pear-shaped flask. The ethyl acetate extract was taken to dryness using a rotary evaporator.

The sample in the pear-shaped flask was taken up in 2.5 ml absolute alcohol and transferred to a stoppered test tube. The remains of the sample in the flask were washed out with

gain of 100 on both the 301 ion and the 280 ion

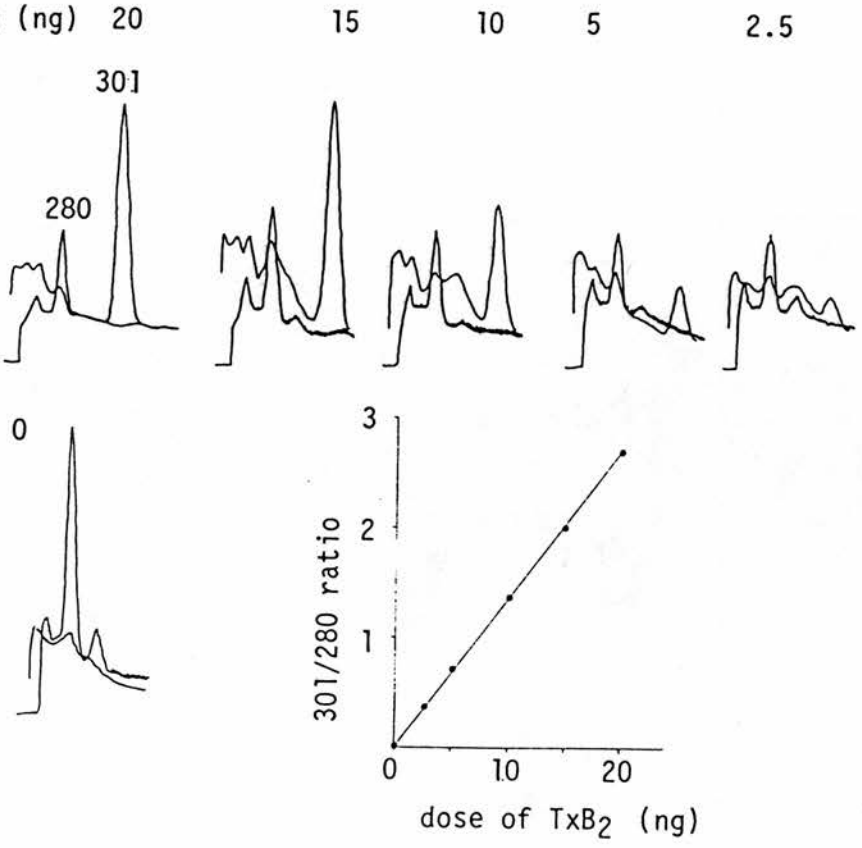


Figure Add.4 Typical GC-MS traces for the TxB_2 standard curve.

2.5 ml distilled water which was added to the alcohol. 5 ml of benzene (BDH Chemicals, Analar quality) was added to the tube. This resulted in an ethanol: water: benzene (1: 1: 2) partition. The tube contents were mixed, then spun in a bench centrifuge to ensure a clear separation between the two layers. The upper benzene layer was removed using a pasteur pipette, and discarded. The ethanol-water layer was transferred back to the original pear-shaped flask and the test tube washed out with a further 2.5 ml ethanol which was transferred again to the flask to give a 67% ethanolic solution. The solution was taken to dryness on the rotary evaporator as before.

The sample was taken up in 2 ml methanol and 10 µl of usually, an, 8-fold dilution was derivatized as for standards and assayed on the GC/MS along with the standards.

For studying the production of TxB2 in the bullock iris sphincter, the preparation was set up as before. 50 µl AA (2mg/ml) was added into a 10 ml organ bath, containing sugar-salt solution. AA produced contraction. After 5 min the bath solution was collected and 2M HCl was added to give a pH of about 3. The rest of the work-up of procedure was the same as for rat PRP.

The results are shown in Figure Add.5. Both rat platelets and bullock iris sphincter can convert AA into TxB2, which indicates that they are able to synthesize TxA2. AA 100 µg/ml was converted by 1 ml rat PRP into 1 µg/ml TxB2 in 30 s, a result similar to that from human PRP (0.2-0.8 µg/ml TxB2 from 100 µg/ml AA) (Jones, R.L., Personal

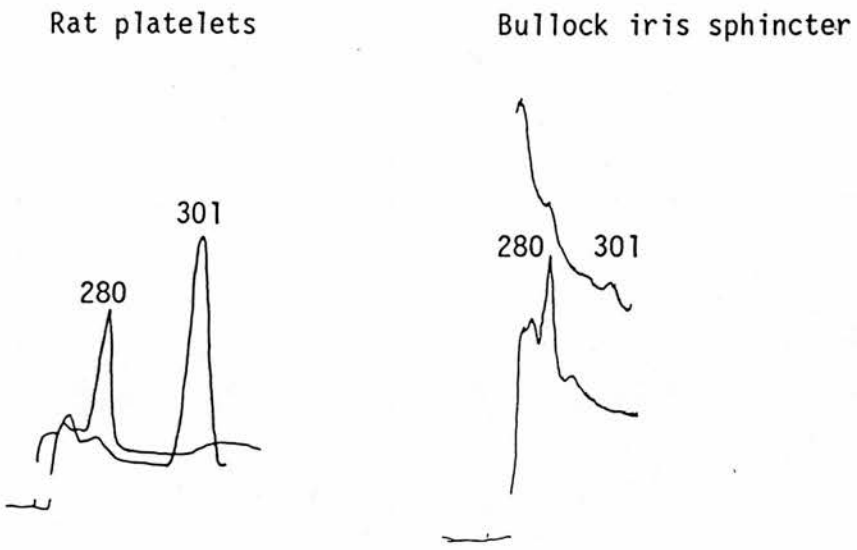


Figure Add.5 TxB_2 generation in the rat platelets and bullock iris sphincter. AA was used as the precursor. The 301 ion peak indicates the existence of TxB_2.

Communication).

Part Two

Reference citations: PP 371 - 384

Section Five

Enhancement by Verapamil of Actions of Prostanoids

on the Rat Anococcygeus Muscle

Reference citations: pp 371 - 384

INTRODUCTION

The rat anococcygeus muscle was originally described by Gillespie (1971, 1972) Gillespie & Maxwell (1971). It consists of parallel bundles of smooth muscles fibres attached to a tendon from the coccygeal vertebrae. Some of these fibres end by merging with the longitudinal muscle of the colon, and other muscle fibres in the male rat continue to end either in the perineum or by forming the retractor penis muscle. The anococcygeus muscles are approximately 3 cm long x0.5 cm wide x130-300 µm thick. Each muscle bundle contains between two and eight fibres with numerous gap junctions between fibres possibly representing regions of electrical continuity. In the narrow tissue clefts between bundles run the autonomic nerves within Schwann cell sheaths. Most of them are adrenergic as shown by their formaldehyde fluorescence, by the presence of dense-cored vesicles within their varicosities, by the ability to enhance these dense cores with 5-HT and their disappearance after chronic treatment with 6-hydroxydopamine or reserpine. Recently varicosities containing larger, electron-opaque vesicles have been found contributing up to 40% of the total and most probably representing the inhibitory nerves (Gibbins & Haller, 1979). A third type of varicosity with mainly clear vesicles accounted for less than 5% of the nerve profiles and may represent a true cholinergic innervation, which does not seem an important neural control as neither contractile nor inhibitory responses to nerve

stimulation are influenced by atropine. The origin of these contractile and inhibitory nerve in the spinal cord has been identified: the contractile nerve is characteristically sympathetic arising in the upper lumbar outflow; the inhibitory equally characteristic, arising from the sacral cord between L5 and S3 (Gillespie & McGrath, 1973). Both are interrupted by ganglia as judged by the action of ganglion blocking agents in vivo.

Its structural arrangement makes the rat anococcygeus muscle an ideal smooth muscle preparations since it fulfils the following criteria:

(1) The preparation consists of a thin layer of smooth muscle cells. This arrangement minimizes the problem of drug access.

(2) Many smooth muscle preparations are derived from circular or helical tissue which introduces difficulties in the recording and interpretation of mechanical responses. As the smooth muscle cells of the rat anococcygeus are arranged longitudinally, this problem does not arise.

(3) The tissue is densely innervated which is an ideal arrangement for studies of noradrenergic transmission.

The preparation has attracted additional interest because it possesses non-adrenergic and non-cholinergic (NANC) nerves mediating inhibitory response as mentioned above.

The electrical properties of the muscle and its response to nerve stimulation have been examined in the isolated preparation. The resting membrane potential is high, about 62 mV, and stable, corresponding to the absence of

spontaneous mechanical activity. Nerve stimulation produces graded depolarizations without spike potentials and these give rise to graded contractions. Guanethidine blocks the adrenergic nerve, depolarises the muscle and raises tone. In the presence of guanethidine nerve stimulation produces mechanical inhibition but little or no hyperpolarization. The responses are different from the large hyperpolarizations produced in the guinea-pig taenia coli by stimulating the non-cholinergic and non-adrenergic nerves, suggesting that the transmitter might also be different.

The muscle is contracted by alpha-adrenergic agents, dopamine, muscarine, 5-HT, vasopressin, PGE1, PGE2 and PGF2a. Beta-adrenergic agents and histamine have no effect on this preparation. The action of ATP is unclear. Little work had been done on prostanoid action in the rat anococcygeus muscle. An attempt was therefore made to define the nature of any prostanoid receptors present. It has been suggested that prostanoids can release calcium from intracellular stores in smooth muscle(Kirtland & Baum, 1972; Malmstrom & Carafoli, 1975; Tsuyoshi & Develin, 1979; van Breemen, Aaronson, Loutzenhiser & Meisheri, 1980; Loutzenhiser & van Breemen, 1981; Brandt, Andersson, Edvinsson & Ljunggren, 1981; Shimizu, Ohta & Toda, 1980). In order to study the relationship between PG actions and calcium movement on this preparation, calcium channel blockers were tried against PGs. Unexpectedly, calcium channel blockers potentiate, rather than inhibit, the action of PGs.

For convenience, the term "calcium antagonists" will be used here to mean both calcium channel blockers and calmodulin antagonists; the reason will be given later.

METHODS

Rat Anococcygeal Preparation

Rats were killed by a blow on the head and bled. The abdomen was opened along the mid-line and the pelvis split. The colon was then cut through at the pelvic brim and the pelvic portion pulled forward. The anococcygeus muscles were separated from surrounding connective tissue, tied and dissected out. The muscle was mounted under 50 mg tension in a 5 ml bath containing Krebs solution at 37°C (unless otherwise stated). The solution wasbubbled with 95%O_2 + 5%CO_2. Changes in tension were measured isometrically with a Grass force displacement transducer (FT 03C) and displayed on a Grass Polygraph. In those experiments when the response to field stimulation was examined the muscles were encircled by a pair of ring platinum electrodes embedded in a plastic tube. A Grass S44 stimulator and timer were used to generate 1 msec pulses at supramaximal voltage at the frequencies given in the text. Responses were measured on a Devices recorder. Pulse trains of 30 s were applied every 4th. min.

In experiments where sympathetic denervation was required 6-hydroxydopamine HBr (250 µg/ml) was added into the organ bath and was washed out after 3 hours. If contractions elicited by adding tyramine (10 µM) were still present, the preparation was incubated for a further 3 hours with the same amount of 6-hydroxydopamine.

Chromatography of urine and hemolysate

Sephadex G50 Fine gel powder (5 g) (Pharmacia Uppsala,

Sweden) was allowed to fully swell in excess distilled water. The Sephadex was added to the water slowly and stirred gently. This formed a dilute slurry which was left to settle. The gel part sank, leaving a clear layer on top. The upper layer was poured off and an equal volume of 4 mM Tris-HCl pH 7.4 was added to form a slurry by stirring gently. The upper layer was poured off and fresh buffer was added again. The procedure was repeated until the upper layer achieved a pH of 7.4. Hibitane 0.002% was added to the slurry to prevent microbial growth.

Plastic pipettes (10 ml) were shortened to 8 cm lengths and fitted with a small plug of quartz wool in the pointed end. Each column was packed with the G50 Fine slurry which had been degassed. 2 ml of 4 mM Tris-HCl buffer (pH 7.4) eluent was pipetted onto the top of the column 5 cm and flow rate estimated by measuring the volume of eluent collected in 1 min. It was about 0.5 ml/min. The column gave a good separation between serum albumin labelled with coomassie blue and procion red.

Urine or hemolysate (1 ml) was laid gently on the drained bed surface and allowed to drain into the bed. The sample which remained on the bed surface and on the column wall was washed into the bed with 1 ml of Tris-HCl buffer eluent, which was allowed to drain away. Then the column was refilled with eluent and samples were collected every 5 min. All the procedures were carried out at room temperature. The swelled gel was stored at 4°C.

Measurement of noradrenaline

The method used was HPLC and electrochemical detection on a carbon paste electrode (Dr. A.G. Watts, unpublished method). The experiments were done with his help.

Owing to the poor solubility of nifedipine in water, 10 mM stock solution of the drug was prepared in absolute ethanol, and diluted 10-fold with distilled water. Because of the known photosensitivity of the dihydropyridines, the experiments with nifedipine were done in the dark.

RESULTS

Effects of Prostanoids

The preparation had neither spontaneous activity nor tone. It contracted to PGE2 and 11,9-epoxymethano PGH2. The threshold concentration for contraction was about 15 ng/ml for both PGE2 and 11,9-epoxymethano PGH2. In Krebs solution 11,9-epoxymethano PGH2 gave a maximum increase in tension of about 5 gram, but PGE2 showed a much lower maximum response. Since the preparation was very readily desensitized by high concentrations of PGE2 and 11,9-epoxymethano PGH2, experiments in this study were carried out with low concentrations of prostanoids. Responses to prostanoids plateaued after 4-10 min remained steady for 2-4 min and then faded gradually. Atropine 1 μM, phentolamine 1 μM and indomethacin 1 μM did not antagonize responses to prostanoids.

The TxA-receptor antagonists EP 045, EP 092 and EP 116 blocked the action of 11,9-epoxymethano PGH2, but not those of PGE2 and noradrenaline. The antagonist was allowed 10 min contact with the tissue before addition of the agonist. Dose ratios are shown in Table A.1 together with the calculated affinity constants.

Some other TxA2 mimetics were also tested. They were EP 011, 15-oxo EP 011, 9,11-azo PGH2, CTA2 and PTA2. EP 011 and 15-oxo EP 011 were about 20 and 10 times more potent than

Table A.1 Affinity constants of TxA_2 antagonists on the rat anococcygeus muscle preparation.

Compound	*Concentration*	*Individual dose-ratios*	*Affinity constant*
EP 045	2.6×10^{-7}	*2.3 2.3 2.4*	$4.9 \pm 0.14 \times 10^{6}\ M^{-1}$ *(n=3)*
EP 092	1.2×10^{-7}	*1.8 2.7 2.8*	$1.2 \pm 0.25 \times 10^{7}\ M^{-1}$ *(n=3)*
	2.4×10^{-7}	*4.3 6.7 10*	$2.5 \pm 0.67 \times 10^{7}\ M^{-1}$ *(n=3)*
EP 116	4.5×10^{-8}	*4.6 4.8 4.9*	$8.3 \pm 0.18 \times 10^{7}\ M^{-1}$ *(n=3)*

11,9-epoxymethano PGH_2 was used as standard agonist.

11,9-epoxymethano PGH2, respectively. 9,11-Azo PGH2 had a potency similar to 11,9-epoxymethano PGH2. CTA2 and 9,11-epoxymethano PGH2 behaved as partial agonists (Figure A.1). PTA2 at 2 µg/ml produced no contractile effect, but it antagonized the action of 11,9-epoxymethano PGH2 and in certain cases it potentiated the action of PGE2 (Figure A.2 p 231.)

Subthreshold concentrations of PGE2 analogues enhanced the contractile effect of TxA2 mimetics. An attempt was made to quantitate this enhancement by calculating a pP2 value. pP2, analogous to pA2, is the negative logarithm of the molar concentration of a potentiating drug which increases the response of half the dose of agonist to that of an initial submaximal dose. The pP2 values of ICI 80205, 16,16-dimethyl PGE2 and PGE2 for potentiation of 11,9-epoxymethano PGH2 action are listed in Table A.2 p 232.

As with the bullock iris sphincter preparation, ZK 36374 was a partial agonist on PGE2 sites on the anococcygeus muscle . It opposed the contractile effect of PGE2 (Figure A.3), but enhanced the contractile effect of 11,9-epoxymethano PGH2.

ICI 81008 produced contractile response with a low maximum (<100 mg), and it did not affect responses to PGE2 or 11,9-epoxymethano PGH2. PGF2a was much weaker than PGE2, but gave a higher maximum than ICI 81008, and enhanced response to 11,9-epoxymethano PGH2. PGI2 and PGD2 were very weak contractile agents.

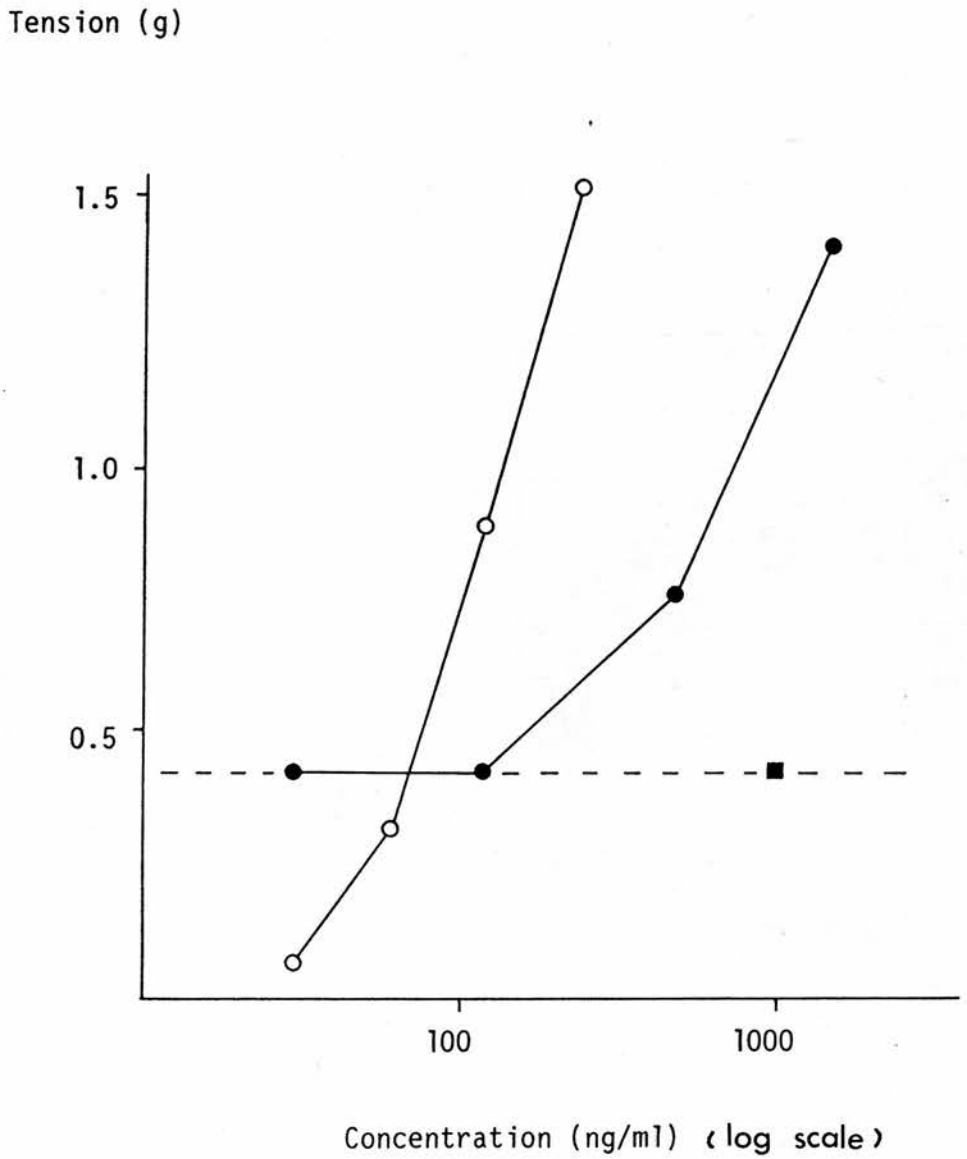


Figure A.1 Rat anococcygeus muscle preparation: partial agonist action of CTA_2; log concentration-response curve for 11,9-epoxy-methano PGH_2 acting alone (open circle) and the corresponding curve (solid circle) in the presence of 1 μg/ml CTA_2 (solid square).

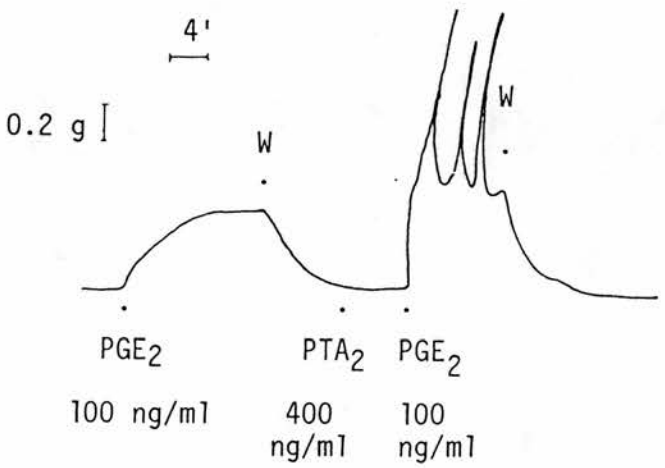


Figure A.2 Rat anococcygeus muscle preparation: interaction of PTA_2 with PGE_2. In the presence of PTA_2, the response to PGE_2 was potentiated, which is shown as the spike contraction.

Table A.2 pP_2 values of PGE_2 analogues for potentiation of the contractile action of 11,9-epoxymethano PGH_2 on the rat anococcygeus muscle preparation.

Compound	pP_2 (mean±s.e.)
(±) ICI 80205	9.0±0.22, n=4
16,16-dimethyl PGE_2	8.4±0.13, n=4
PGE_2	7.6±0.040, n=3

Subthreshold concentrations for contraction were used for all the PGE_2 analogues.

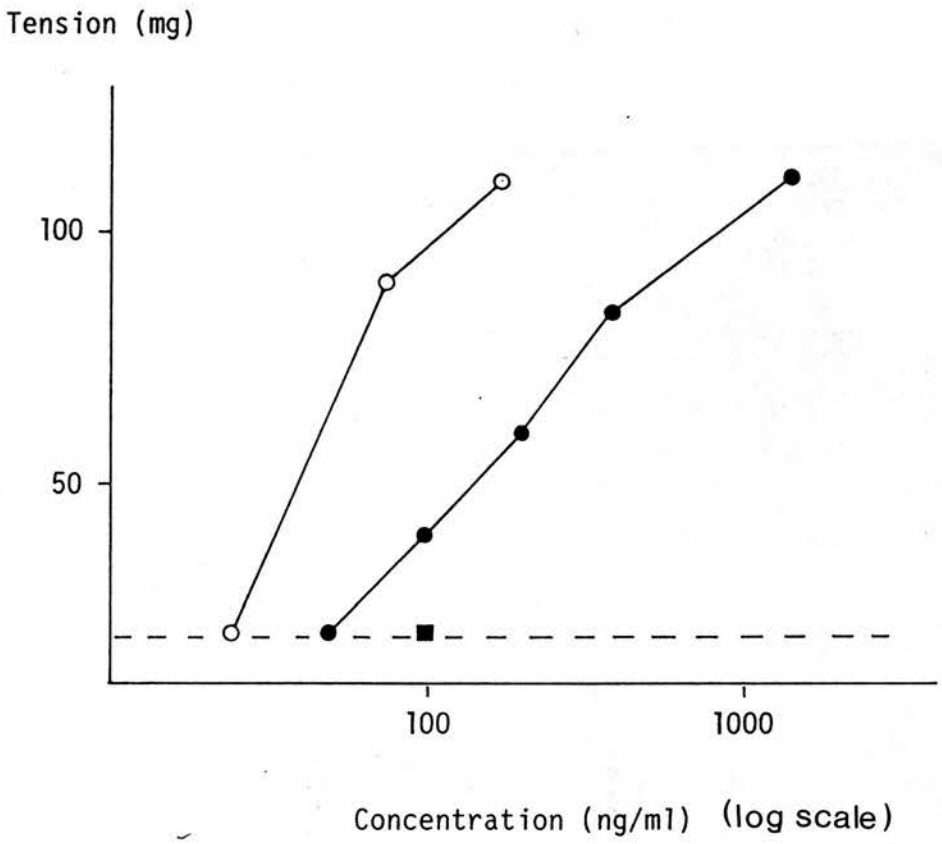


Figure A.3 Rat anococcygeus muscle preparation: interaction of ZK 36374 with PGE_2; log concentration-response curve for PGE_2 acting alone (open circle) and the corresponding curve (solid circle) in the presence of 100 ng/ml ZK 36374 (solid square).

Effects of Calcium Antagonists on Prostanoids and Noradrenaline

The effect of verapamil on the response to PGs was studied. Verapamil was allowed 5 min contact with the preparation before adding agonists. Surprisingly, verapamil (1.0-50 μM) enhanced the contractile effects of 11,9-epoxymethano PGH2, 9,11-azo PGH2, PGE2 and PGF2a (Figure A.4), but not ICI 81008. In contrast, contractions elicited by noradrenaline, potassium or carbachol were inhibited by verapamil. Indomethacin (1.0 μM), atropine (1.0 μM) or phentolamine (1.0 μM) had no effect on the enhancement.

6-Hydroxydopamine was used in vitro to produce sympathetic denervation chemically. This procedure was without effect on the potentiating action of verapamil on 11,9-epoxymethano PGH2. pP2 values for control and treated preparations were 5.03 and 5.23, respectively.

Some other drugs were also tested for the potentiating activities. The results are listed in Table A.3. Difficulties were encountered with the dissolution of pimozide. It could be dissolved in 40% acetic acid to make a 10 mM stock solution. High concentration (0.1 mM) of pimozide in this form elicited contraction and potentiated the action of 11.9-epoxymethano PGH2. 40% acetic acid diluted appropriately gave similar effects. Pimozide was also dissolved in 50% alcohol with 5-6 drops 1N HCl to made 10 mM stock solution. The solution was added to the organ bath to give a final concentration of 20 μM pimozide. In

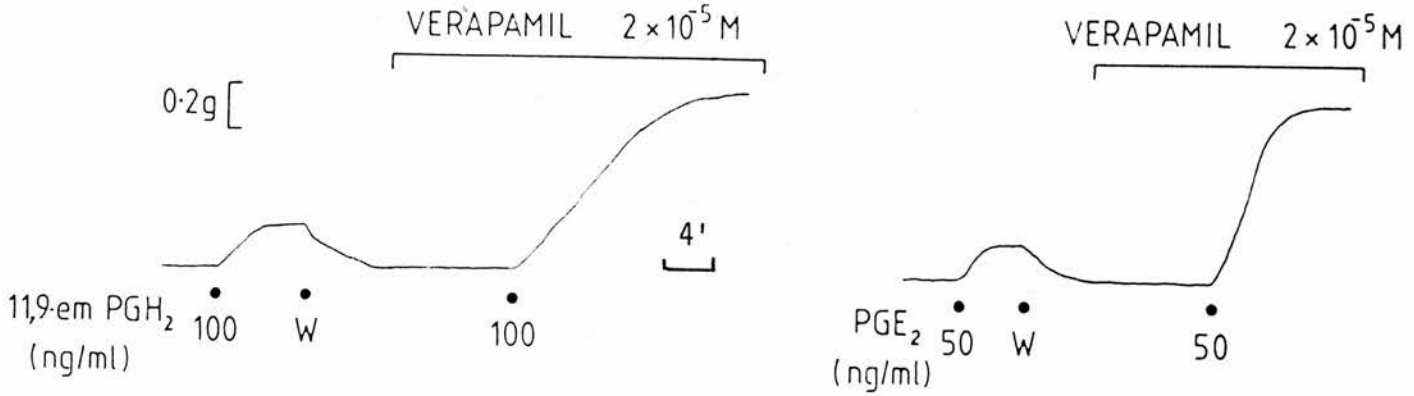


Figure A.4 Rat anococcygeus muscle preparation: potentiating effect of verapamil on the response to 11,9-epoxymethano PGH_2 or PGE_2.

Table A.3 pP_2 values (mean±s.e., n=4) for potentiation of the contractile action of 11,9-epoxymethano PGH_2 on the rat anococcygeus muscle preparation.

Compound	pP_2	Compound	pP_2
trifluoperazine	5.88 ± 0.07	propranolol	4.38 ± 0.11
amitriptyline	5.58 ± 0.21	penfluridol	4.30 ± 0.12
promethazine	5.39 ± 0.23	trifluoperazine sulphoxide	4.29 ± 0.15
verapamil	5.03 ± 0.19	trans-flupenthixol	4.01 ± 0.04
chlorpromazine	4.94 ± 0.02	D 600	3.83 ± 0.12
cis-flupenthixol	4.48 ± 0.10		

this case the vehicle had weak inhibitory effect and pimozide produced no effect. Nifedipine and flunarizine showed minimal potentiation at 10 μM and inhibited 11,9-epoxymethano PGH2-produced contractions at 100 μM. Xylocaine at 100 μM inhibited contraction due to 11,9-epoxymethano PGH2.

The potentiating activities of some drugs on response to PGE2 are shown in Table A.4.

Both noradrenaline and carbachol contracted this preparation. With noradrenaline which produced tonic responses at concentrations higher than 1.0 μM, verapamil, D 600, nifedipine and trifluoperazine produced inhibitory effects. The pA2 values were 6.10(± s.e.0.07, n=7) for verapamil, 5.09(± s.e.0.063, n=7) for D 600, 4.68(± s.e.0.32, n=4) for nifedipine and 5.16(± s.e.0.26, n=3) for trifluoperazine.

It is noteworthy that after the development of contraction induced by 11,9-epoxymethano PGH2, addition of 100 μM verapamil would cause dual response: an increase in tension followed by decrease in tension (Figure A.5).

Effect of low temperature on the enhancement.

In a series of experiments, the bath temperature was set at 12°C. At this low temperature PGE2 activity was reduced remarkably, but the potentiating activity of PGE2 was unchanged. The pP2 value was 8.06(± s.e.0.17, n=3) for potentiating 11,9-epoxymethano PGH2. On the other hand the potentiating activities of other drugs were reduced to a

Table A.4 pP_2 values for potentiation of the contractile action of PGE_2 on the rat anococcygeus muscle preparation.

Compound	pP_2 (mean±s.e.)
promethazine	6.5±0.13, n=3
chlorpromazine	6.2±0.21, n=4
trifluoperazine	6.1 6.0
verapamil	5.0±0.19, n=4
propranolol	5.3±0.18, n=3
phentolamine	4.9 5.3
D 600	4.2±0.064, n=3

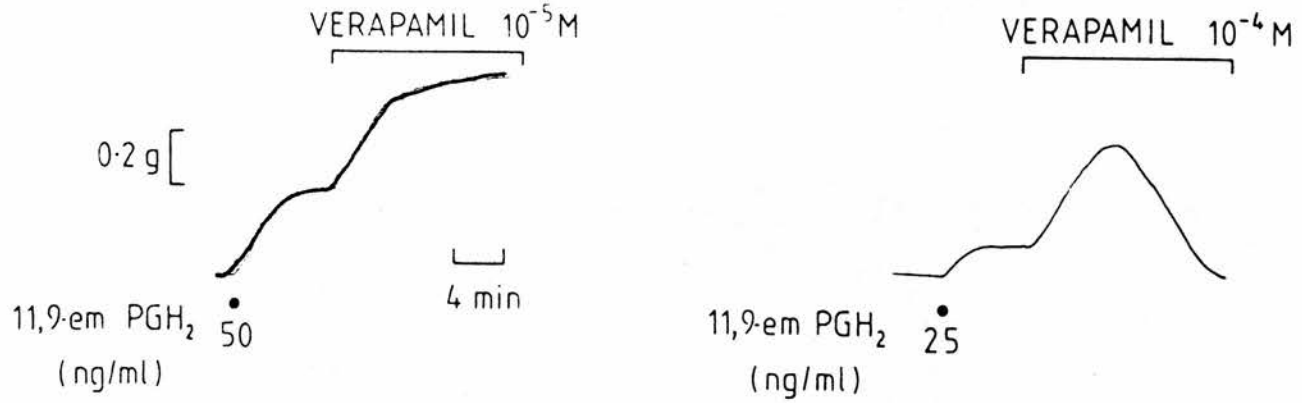


Figure A.5 Rat anococcygeus muscle preparation: adding verapamil 10^{-5} M to an established submaximal response to 11,9-epoxymethano PGH_2 produces an additional contraction (left). The same effect is seen with 10^{-4} M verapamil, except that the contraction fades after about 5 min (right).

great extent. The pP2 value of verapamil for potentiation of 11.9-epoxymethano PGH2 was 4.22 and the pP2 value of chlorpromazine for potentiation of PGE2 was 4.70.

Effect of calcium on the enhancement.

The sensitivity of the preparation to 11,9-epoxymethano PGH2 was found to be little affected by changes in external calcium concentration over the range 0.6-5.0 mM, but preincubating the preparation in a nominally calcium free solution for 30 min reduced the maximum response by about 90%. The potentiating activity of verapamil against 11,9-epoxymethano PGH2 was measured in Krebs solution with different calcium concentrations. The results are shown in Table A.5. In the absence of calcium in the bathing medium, verapamil still enhanced the contraction to 11,9-epoxymethano PGH2, albeit to a limited extent.

In the absence of calcium noradrenaline at higher concentrations produced contractile response and the response was not affected by 1.0-20 μM verapamil, but suppressed by 100 μM verapamil (Figure A.6).

Effects of Trypsin on Prostanoids and Noradrenaline Actions.

A fixed concentration of 11,9-epoxymethano PGH2 or noradrenaline was added to obtain a response. Following wash-out trypsin 40 μg/ml was added into the organ bath, kept for 2-4 min and washed out. Then the preparation was challenged with the same amount of 11,9-epoxymethano PGH2 or noradrenaline. The results are shown in Figure A.7a and b

Table A.5 pP_2 values of verapamil against 11,9-epoxymethano PGH_2 at different calcium concentrations on the rat anococcygeus muscle.

Calcium concentration (mM)	pP_2 (mean±s.e.)
0.6	5.2±0.15, n=3
1.2	5.6±0.098, n=3
2.5	5.0±0.19, n=4
5.0	5.0

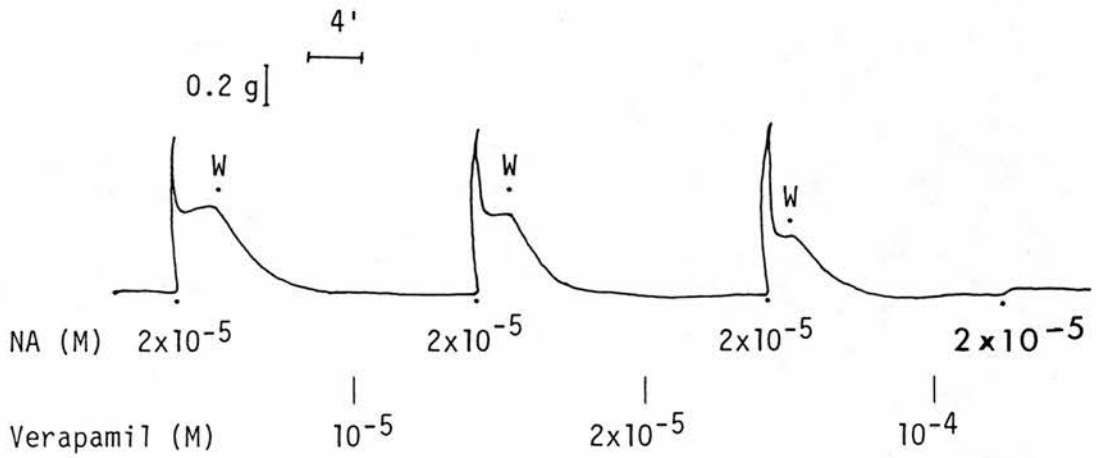


Figure A.6 Rat anococcygeus muscle preparation: effects of verapamil on the response to noradrenaline in the absence of calcium.
NA = noradrenaline, W = wash.

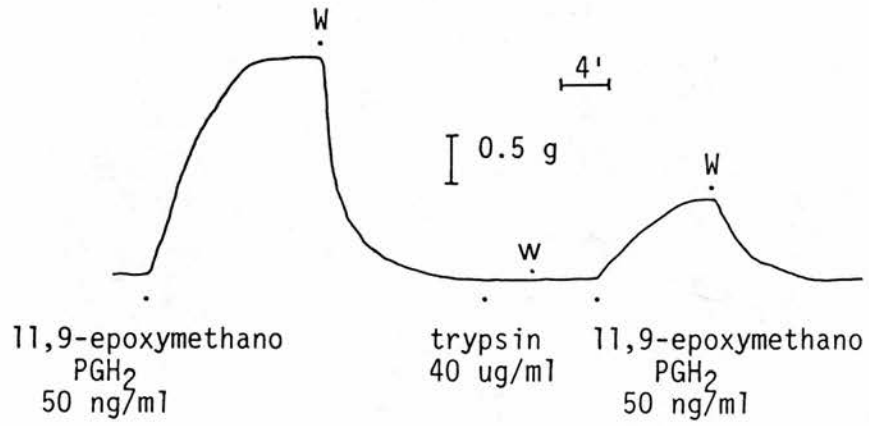


Figure A.7a Rat anococcygeus muscle preparation: effects of trypsin on the response to 11,9-epoxymethano PGH_2.
W = wash.

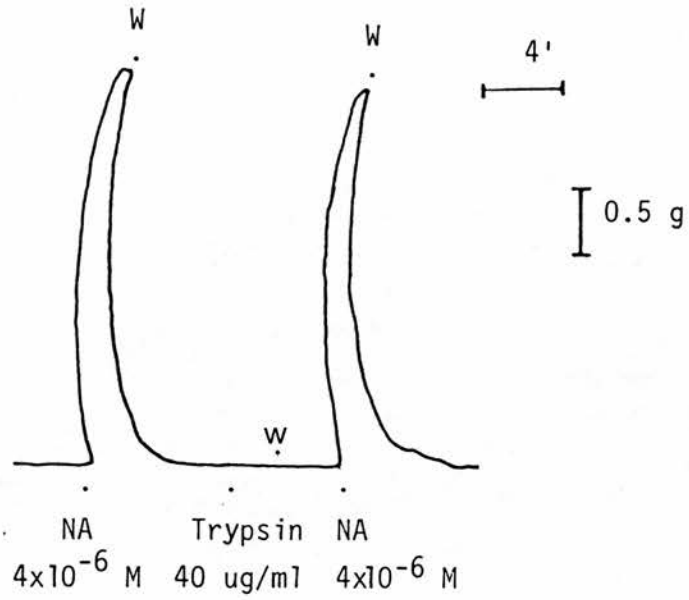


Figure A.7b Rat anococcygeus muscle preparation: effects of trypsin on the response to noradrenaline. NA = noradrenaline, W = wash.

Pretreatment of the preparation with trypsin diminished responses to 11,9-epoxymethano PGH2, but not to noradrenaline.

Effect of Sodium Azide (NaN_3) on Prostanoid and Noradrenaline Action.

In the presence of 10 μM NaN_3 contractions to 11,9-epoxymethano PGH2 and to noradrenaline were inhibited markedly. When NaN_3 was washed out sensitivity to both 11,9-epoxymethano PGH2 and noradrenaline was restored.

Effect of Urine and Hemolysate on Prostanoid and Noradrenaline Action.

It was discovered by chance that rat urine collected from rat bladder, augmented contractile action of 11,9-epoxymethano PGH2. Carnosine and urea were tested. The former substance at 200 μM showed no effect, the latter at 4% w/v produced inhibitory action on 11,9-epoxymethano PGH2-contracted preparation.

Chromatography p 224 was then used to effect a crude separation of the elements in rat or human urine. The yellow-coloured fraction (YCF) in both rat and human urine showed potentiating activity.

Rat red blood cells, obtained after rat PRP was collected in rat platelet experiments were also examined. The intact red blood cell produced no effect, but the hemolysate enhanced

contraction due to 11,9-epoxymethano PGH2. Again the hemolysate was chromatographed. The red-coloured fraction (RCF) in the hemolysate possessed potentiating activity. Purchased hemoglobin (containing MHb 75%) and methemoglobin(MHb) showed the same potentiating activity. Their pP2 values were 4.19(± s.e.0.11, n=3) for hemoglobin (molecular weight 64,500) and 4.11(± s.e.0.081, n=3) for methemoglobin. Hemin was dissolved in 80% alcohol to make 10 mM solution and further diluted with 0.9% NaCl. Hemin at concentrations less than 10 μM produced no effect, at 100 μM it showed solvent effect.

RCF, YCF, hemoglobin and methemoglobin produced no effect on response to noradrenaline.

Effects of Calcium Channel Blockers on Contractions due to High Potassium.

In this series of experiments 118 mM NaCl in Krebs solution was replaced by equal molar concentration of KCl. The high potassium elicited a biphasic contraction of the rat anococcygeus muscle. Immediately after addition, a large contraction corresponding to an increase in tension of about 5.0-7.0 G was seen. The response peaked at 2-4 min, then faded to a lower level, followed by a second rise in tone (tonic contraction). The preparation was allowed 2-3 hours to achieve a stable tone with wash-out from time to time. The wash-out gave rise to a fall in tone. Between wash-outs, the bath solution was collected, and its noradrenaline content was measured. It was estimated that the output of noradrenaline was approximately 15 ng/mg wet tissue/min at

the beginning and 0.05 ng/mg wet tissue/min after 2 hours. Noradrenaline which remained in the tissue after the 2 hour incubation was 1 ng/mg wet tissue. When the tone became stable, the experiments was conducted as follows: calcium was withdrawn from the high K^+ Krebs solution, and after the muscle tone had returned to a basal level, calcium was added cumulatively to the organ bath to give dose-dependent contractions. Following wash-out, a calcium channel blocker was added and 10 min later another calcium concentration-response relationship was established. It should be mentioned that there was a small rise in tension resistant to the calcium removal.

The contractile response to calcium in the presence of high K^+ was highly reproducible. Figure A.8 shows calcium concentration-response curve alone and in the presence of a fixed concentration of verapamil. Increasing the concentration of calcium overcame the blockade produced by verapamil. Higher concentrations of calcium were not tested since the bathing solution become cloudy with concentrations of calcium more than 10 mM.

The calcium-produced contraction was blocked to greater extents as the concentration of calcium channel blocker was increased. These results are shown with different concentrations of calcium in Figure A.9. Verapamil was the most potent blocking agent among the calcium channel blockers tested. pA2 values were 6.30 for verapamil, 6.15 for D 600, 6.00 for trifluoperazine, 5.00 for nifedinpine and 4.00 for flunarizine. Trifluoperazine, phentolamine and

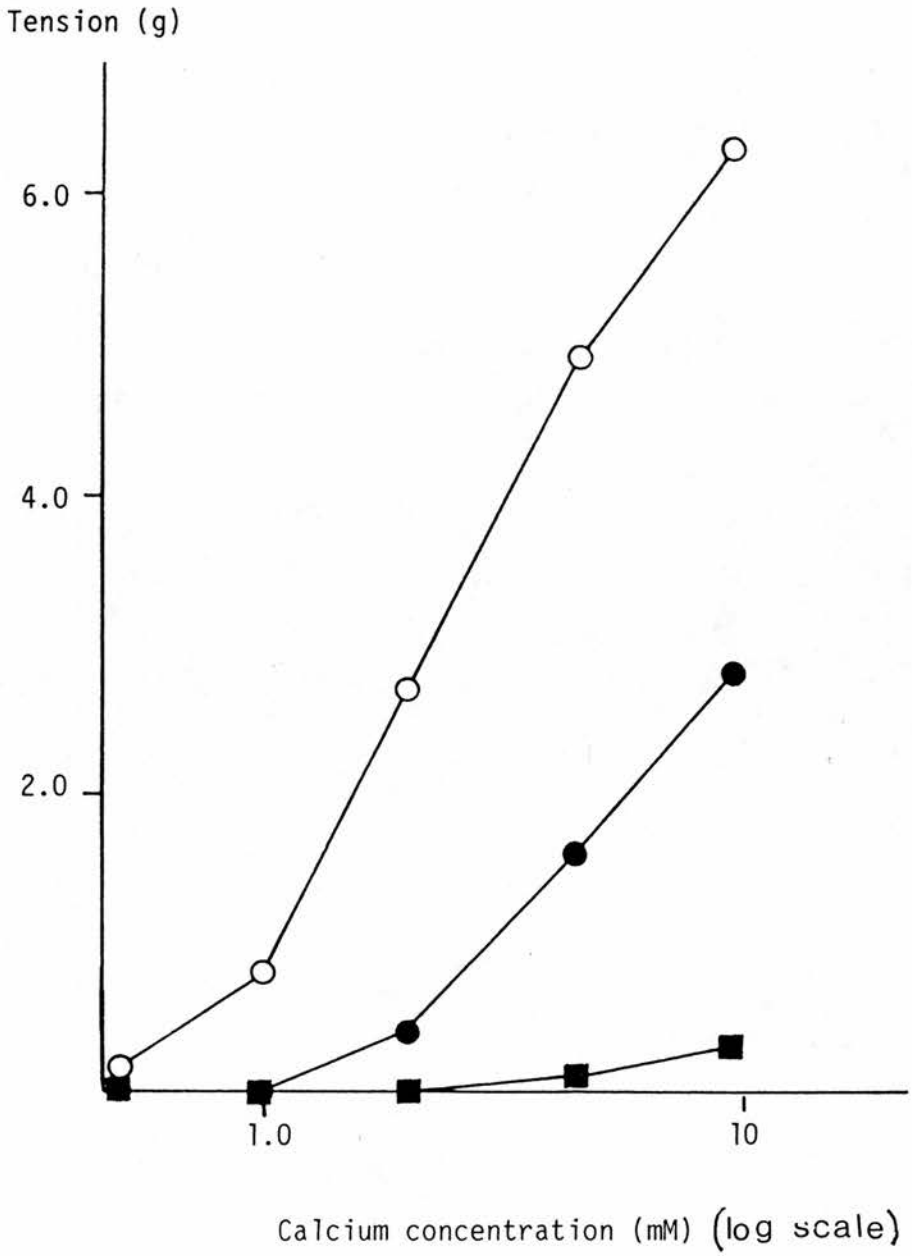


Figure A.8 Rat anococcygeus muscle preparation: effects of verapamil on the contractile response to calcium in the presence of high potassium. Open circle indicates calcium acting alone, solid circle indicates calcium acting in the presence of 5×10^{-7} M verapamil, and solid square in the presence of 10^{-6} M verapamil. Verapamil was allowed 10 min contact with the tissue before adding calcium.

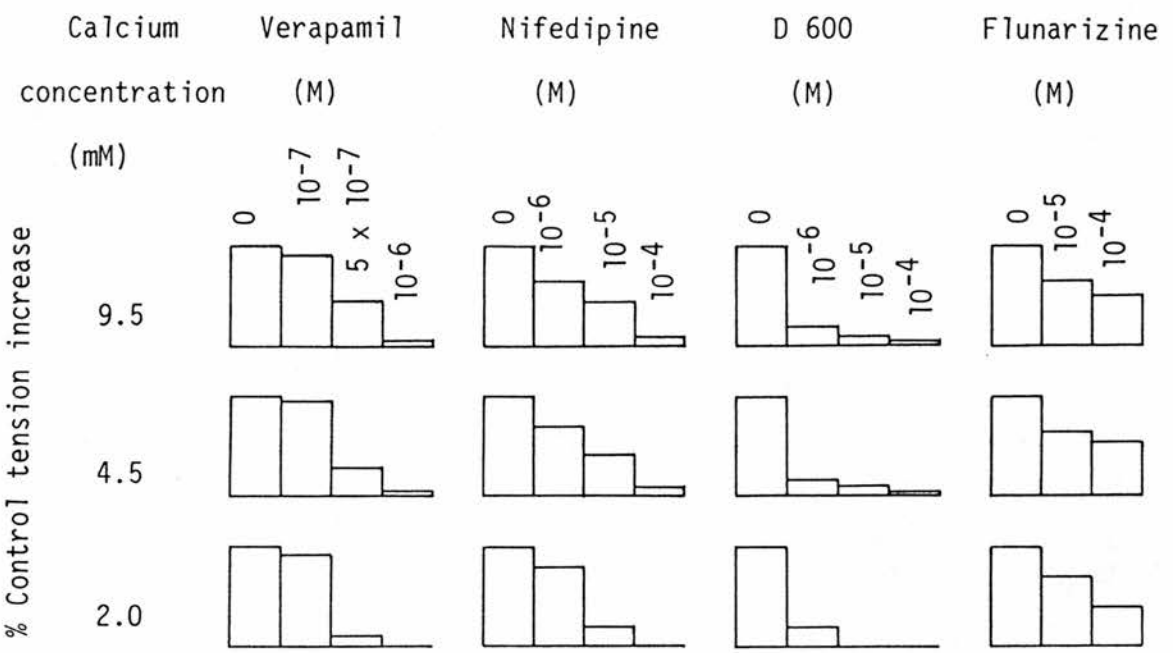


Figure A.9 Rat anococcygeus muscle preparations: effects of calcium channel blockers on calcium-induced contraction in the presence of high potassium. The response to a concentration of calcium in the presence of different concentrations of a calcium channel blocker was compared with the response to the same concentration of calcium in the absence of the calcium channel blocker. Calcium channel blockers were allowed 10 min contact with the tissues before adding calcium. Control experiments were performed first, and the control tension increase was taken as 100%.

propranolol also blocked the potassium-produced contraction. Figure A.10 shows responses to calcium in the presence and absence of a fixed concentration of trifluoperazine in comparison with calcium channel blockers. Trifluoperazine was less active than D 600 in inhibiting high potassium-produced contraction.

Effects of PGE2 and 11,9-Epoxymethano PGH2 on Field Stimulation

Contractile response to field stimulation.

Field stimulation produced contractile responses both at frequencies of 5 Hz and 20 Hz. Since field stimulation at 20 Hz gave higher contraction this frequency (for 30 s, every 4th min) was chosen for studying the contractile response due to field stimulation. The response was invariably potentiated by subthreshold concentrations of 11,9-epoxymethano PGH2 (Figure A.11) and the potentiation was not affected by atropine. The action of PGE2 on the response to field stimulation was variable. In some cases subthreshold concentrations of PGE2 inhibited the response, but in other cases potentiated it (Figure A.12).

In order to find out whether the prostanoids act postsynaptically or presynaptically, effects of the prostanoids on noradrenaline were investigated. Subthreshold concentrations of both PGE2 and 11,9-epoxymethano PGH2 augmented responses to noradrenaline (Figure A.13a & b).

Inhibitory response to field stimulation.

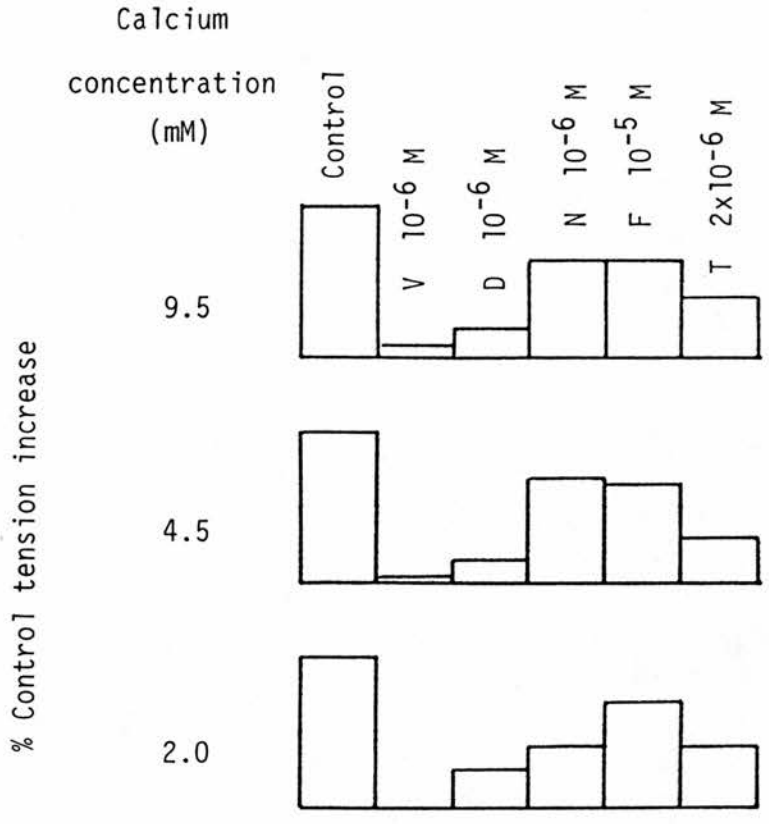


Figure A.10 Rat anococcygeus muscle preparations: Comparison of potency of calcium antagonists. The experiments were conducted in different preparations with different calcium channel blockers. The response to a calcium antagonist was compared with its own control on the same preparation. The control experiments were done first. The control tension increase was taken as 100%. Calcium antagonists were allowed 10 min contact with the tissues before adding calcium. All experiments were done in high potassium Krebs' solution containing 2.0, 4.5 or 9.5 mM calcium. V = verapamil, D = D 600, N = nifedipine, F = flunarizine, T = trifluoperazine.

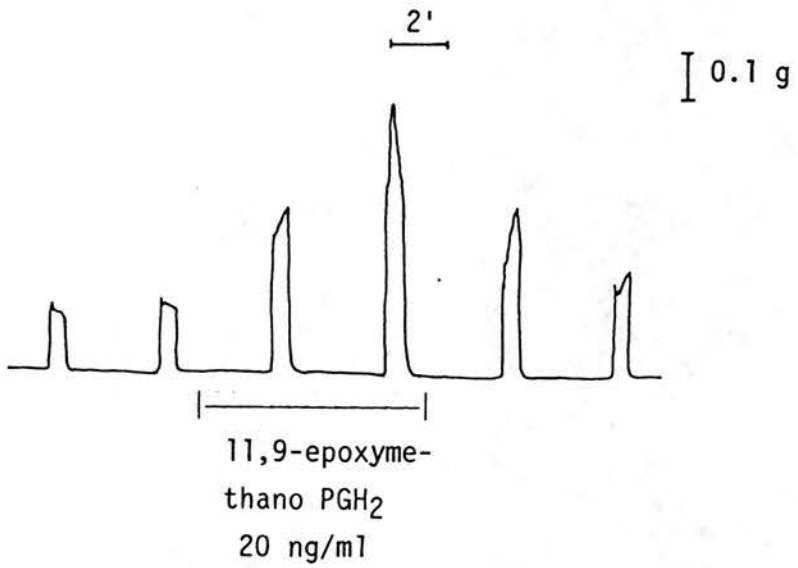


Figure A.11 Rat anococcygeus muscle preparation: potentiating effects of 11,9-epoxymethano PGH_2 on the response to field stimulation at 20 Hz and 30 V.

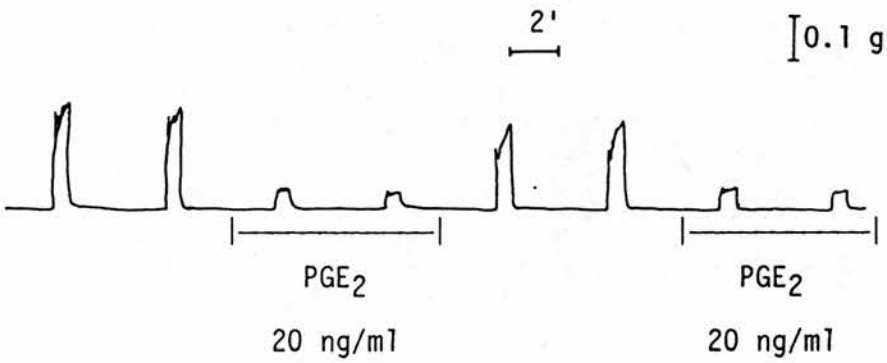


Figure A.12 Rat anococcygeus muscle preparation: inhibitory effects of PGE_2 on the response to field stimulation at 20 Hz and 30 V.

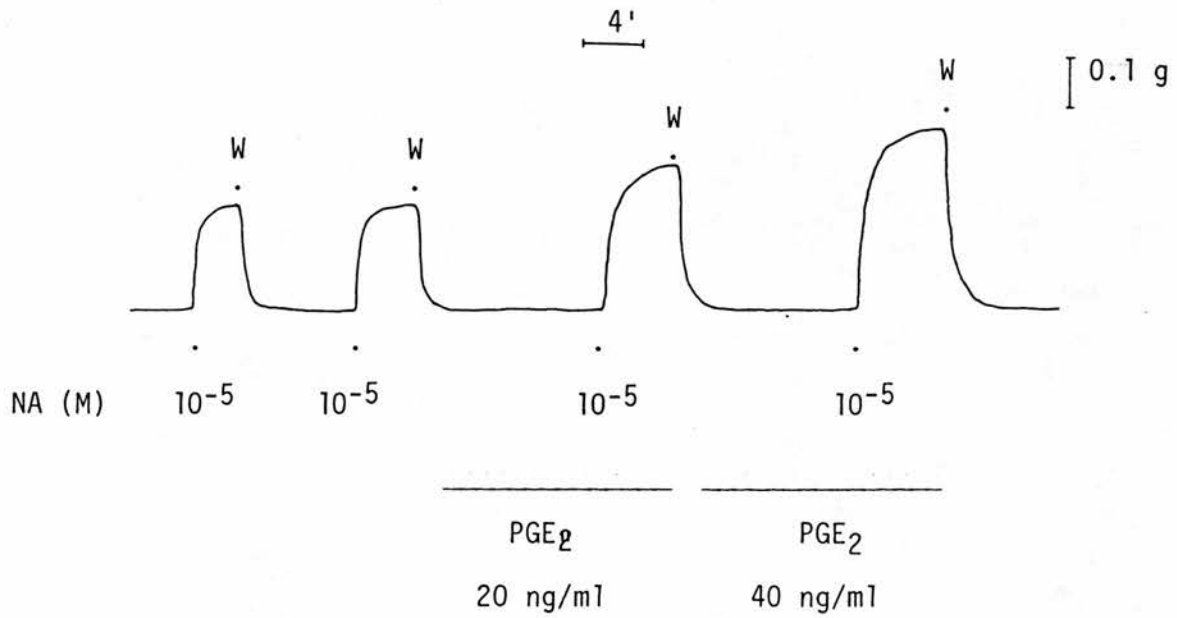


Figure A.13a Rat anococcygeus muscle preparation: potentiating effects of PGE_2 on the response to noradrenaline. NA = noradrenaline, W = wash.

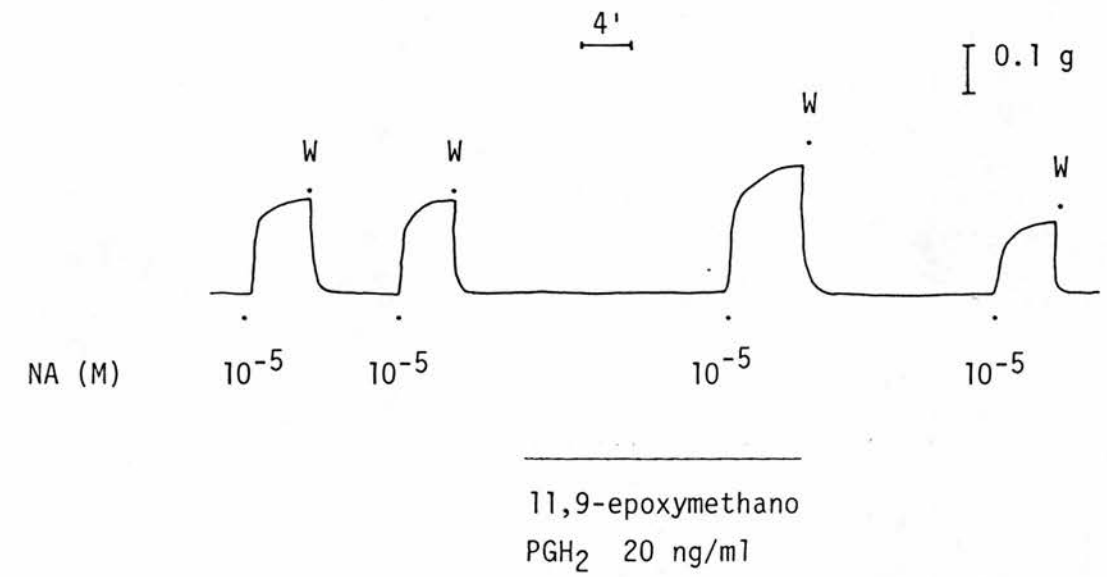


Figure A.13b Rat anococcygeus muscle preparation: potentiating effects of 11,9-epoxymethano PGH_2 on the response to noradrenaline.
NA = noradrenaline, W = wash.

For investigating effects of prostanoids on inhibitory responses to field stimulation a frequency 5 Hz at 110 V was chosen. Tone was provided with PGE2, 11,9-epoxymethano PGH2 or guanethidine.

In calcium free Krebs solution neither contractile nor relaxant responses to nerve stimulation could be seen. At low temperature (12°C) the inhibitory response was markedly reduced.

In this study guanethidine at 20 μM abolished the motor response to field stimulation without raising the resting tone of the muscle. If guanethidine was applied initially at a concentration of 40 μM the anococcygeus muscle showed a rise in tone and responses to field stimulation became biphasic initially, progressing to purely inhibitory responses as the tone continued to rise (Figure A.14). Phentolamine 1.0 μM inhibited the contractile responses to field stimulation partly.

With PGE2 the field stimulation gave a relaxant response and verapamil (10 μM) did not block the response (Figure A.15).

When tone was provided with 11,9-epoxymethano PGH2, the field stimulation caused either a contractile response or a biphasic response. inhibition followed by contraction or contraction followed by inhibition. Atropine at 2.0 μM or indomethacin at 2.0 μM failed to block the contractile component(Figure A.16a). Adding guanethidine 20 μM or phentolamine 20 μM converted the contractile or biphasic response into an inhibitory one (Figure A.16b).

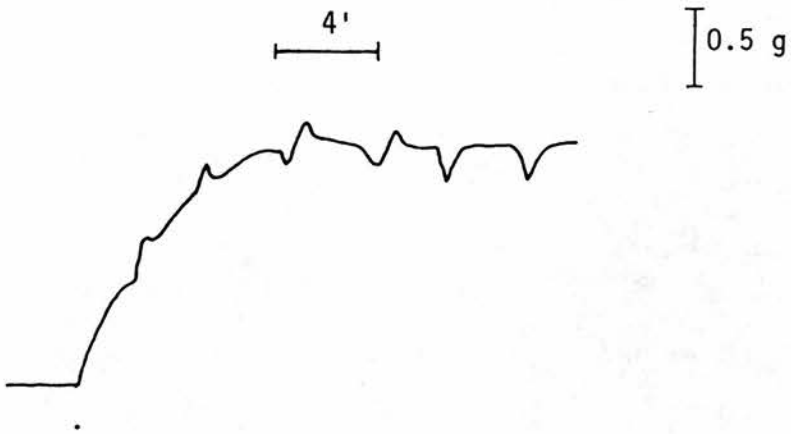


Figure A.14 Rat anococcygeus muscle preparation: responses to field stimulation at 5 Hz and 120 V when tone was provided with 40 μM guanethidine.

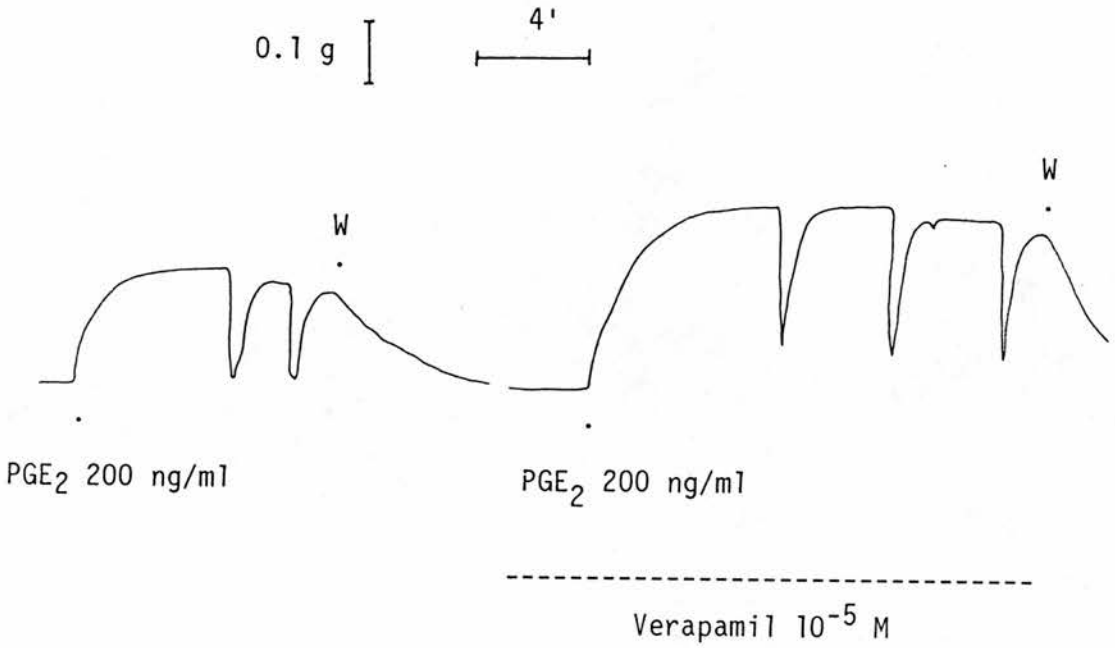


Figure A.15 Rat anococcygeus muscle preparation: the effect of verapamil on the inhibitory response to field stimulation at 5 Hz and 110 V when tone was provided with PGE_2. W = wash.

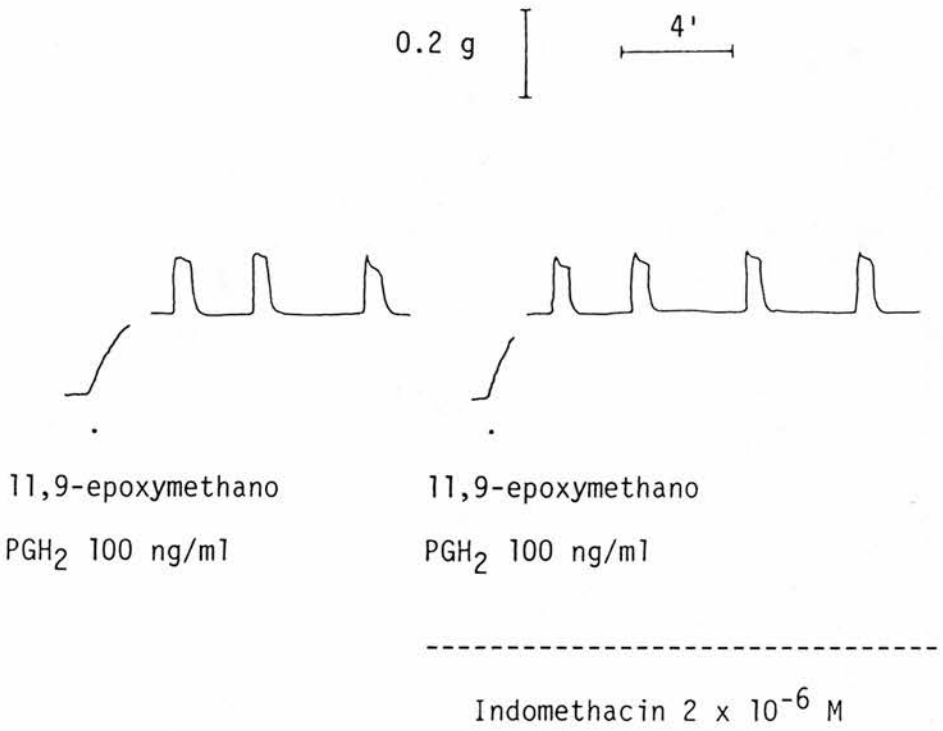
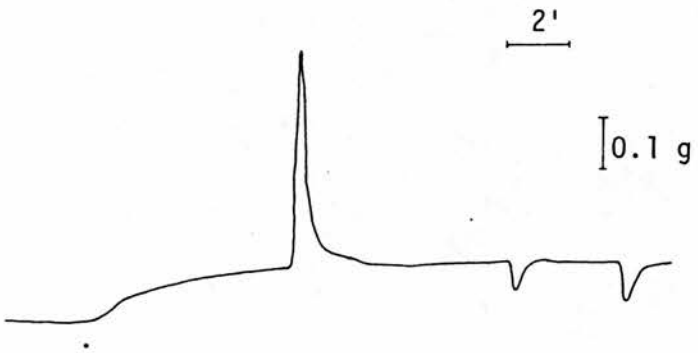


Figure A.16a Rat anococcygeus preparation: the effect of indomethacin on responses to field stimulation at 20 Hz and 50 V when tone was provided with 11,9-epoxymethano PGH_2.



11,9-epoxymethano PGH_2

40 ng/ml

Guanethidine

20 uM

Figure A.16b Rat anococcygeus muscle preparation: the reversal by guanethidine of responses to field stimulation at 5 Hz and 110 V when tone was provided with 11,9-epoxymethano PGH_2.

Chlorpromazine at 10 μM, and promethazine at 10 μM had no effect on the inhibitory response produced by the field stimulation in the presence of guanethidine or 11,9-epoxymethano PGH2, but propranolol 100 μM and verapamil 20 μM reduced the inhibitory response (Figure A.17a, b & c).

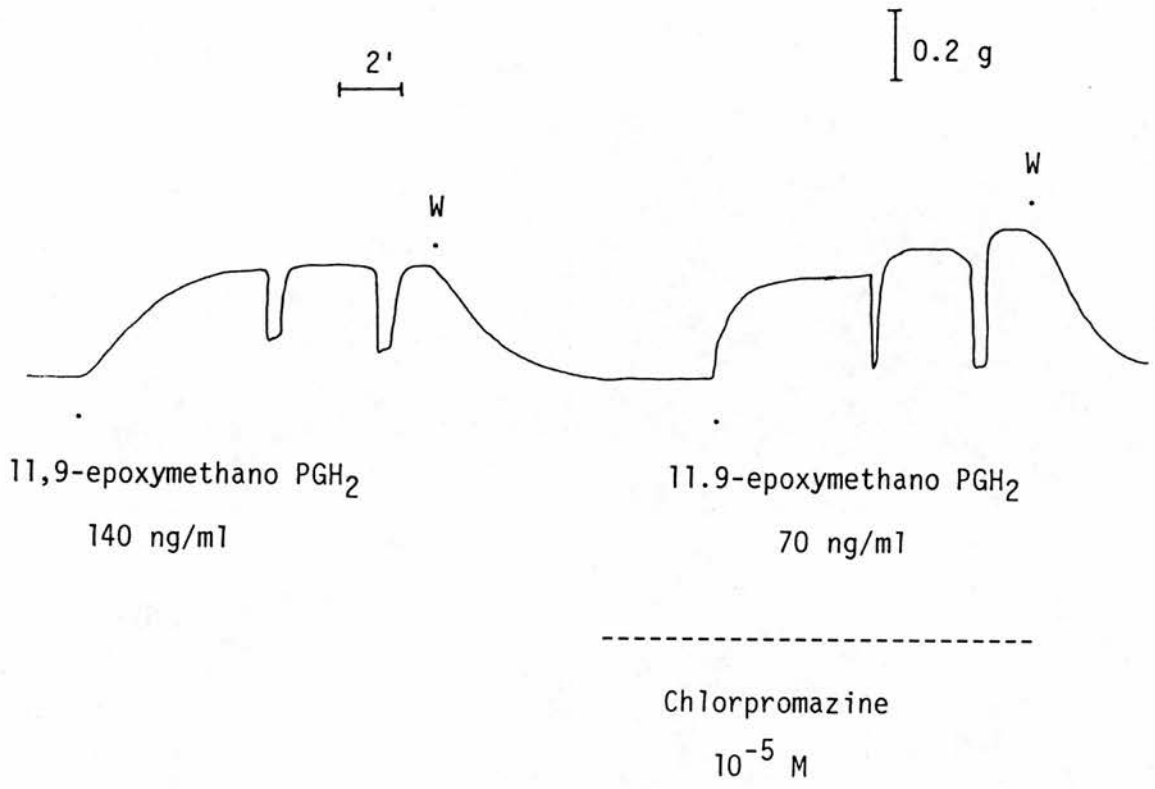


Figure A.17a Rat anococcygeus muscle preparation: the effect of chlorpromazine on the inhibitory response to the field stimulation at 5 Hz and 110 V when tone was provided with 11,9-epoxymethano PGH_2. W = wash.

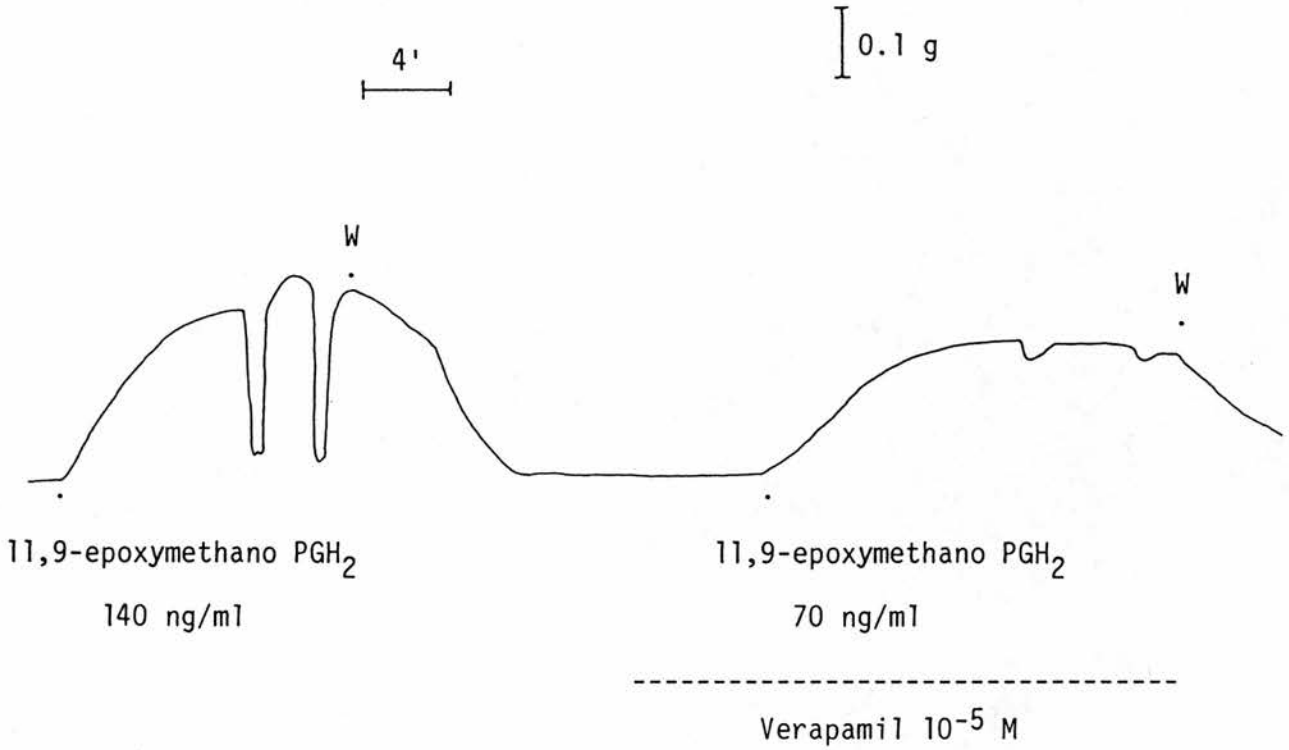


Figure A.17b Rat anococcygeus muscle preparation: the effect of verapamil on the inhibitory response to the field stimulation at 5 Hz and 110 V when tone was provided with 11,9-epoxymethano PGH_2. W = wash.

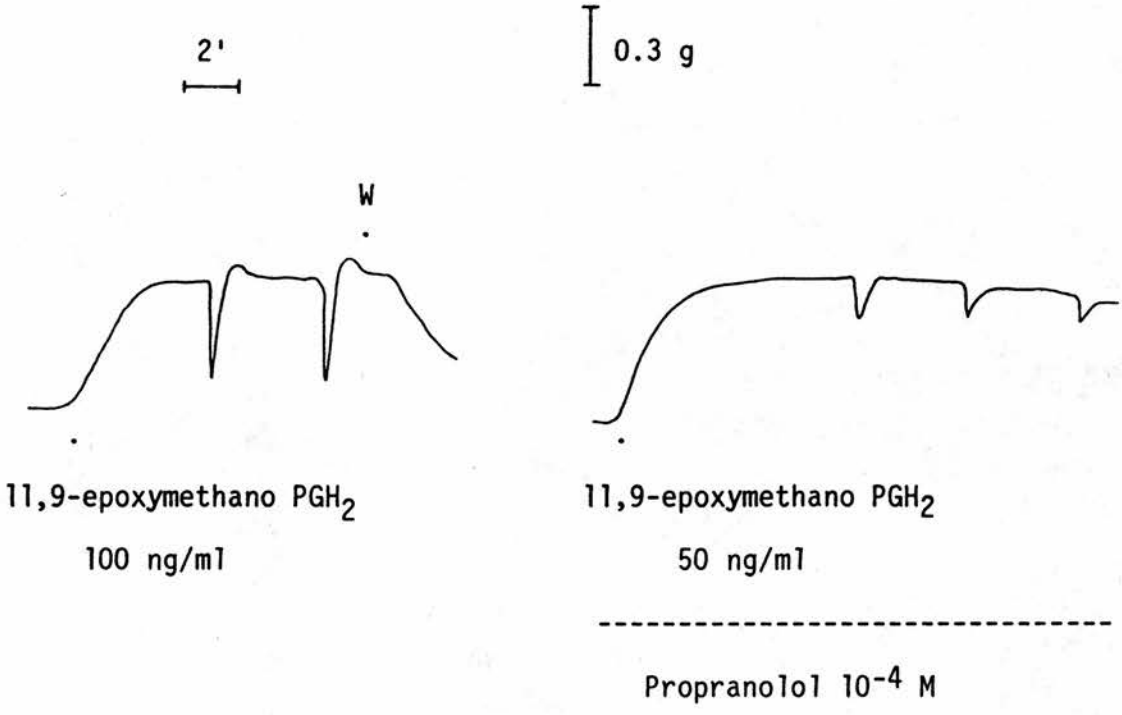


Figure A.17c Rat anococcygeus muscle preparation: the effect of propranolol on the inhibitory response to field stimulation at 5 Hz and 110 V when tone was provided with 11,9-epoxymethano PGH_2. W = wash.

DISCUSSION

The rat anococcygeus muscle contains PGE2 and TxA2 receptors, and probably PGF2a receptors.

The PGE2 receptor system is characterized by the high activity of PGE2, the partial agonism of ZK 36374 and the potentiating action of PGE2 analogues on the response to 11,9-epoxymethano PGH2. The order of potency for the potentiation is ICI 80205> 16,16-dimethyl PGE2> PGE2, as found in the bullock iris sphincter and rat gastric fundus for contractile activity.

The TxA2 receptor sites are sensitive to 11,9-epoxymethano PGH2 and susceptible to blockade by TxA2 antagonists. Unlike in the bullock iris sphincter, CTA2 was weaker than 11,9-epoxymethano PGH2 and showed partial agonism at thromboxane receptors in the rat anococcygeus muscle. PTA2 showed no contractile effect on its own and blocked the action of 11.9-epoxymethano PGH2. PTA2 may not be a pure antagonist on this preparation since it could potentiate the action of PGE2.

It is probable that there exists a third group of prostanoid receptor ---- PGF2a receptor sites. ICI 81008, a specific PGF2a mimetic, showed properties distinct from PGE2, PGF2a and 11,9-epoxymethano PGH2. It did not potentiate 11,9-epoxymethano PGH2 and its contractile action was not augmented by verapamil. Although PGF2a potentiated response to 11,9-epoxymethano PGH2 and its action was enhanced by

verapamil, this could be explained by its action on PGE2 receptor sites, as confirmed in the bullock iris sphincter preparation.

Cellular Calcium Metabolism.

In order to understand the unexpected effect of calcium antagonists at the rat anococcygeus muscle, it is useful to present a synopsis of normal cellular calcium metabolism first.

A 100 years ago Sidney Ringer discovered the vital role of calcium in the maintenance of cardiac contractility. Since then it has been found that intracellular free calcium plays a role as the regulator for numerous different types of cell function throughout the animal and plant kingdoms. Not all these cell functions and their regulatory mechanisms are so well worked out as is the case with muscular contraction, where contraction is both triggered and modulated by the amount of free calcium within the cell. However, calcium is known to be involved in blood clotting and coagulation, cellular adhesion and integrity and membrane stability, bone and teeth formation, enzyme activity, control of certain aspects of cyclic nucleotide metabolism, mediation of some actions of prostaglandins, glandular and other cellular secretory formation, neuronal transmission, in cell transformation and division, in transducing sensory signals, in the production of free radicals by activated polymorphs, in coupling energy

production from intermediary metabolism to energy needs and in the control of membrane ion permeabilities, gravitropism in plants, and undoubtedly many more physiological functions. Calcium also mediates the pharmacological and toxicological actions of numerous drugs and chemicals. However the messenger function of calcium has been appreciated only comparatively recently (Kretsinger,1971 & 1976; Cheung, 1980, 1982 & 1983). This messenger function for calcium is made possible by three key features of cellular calcium regulation: (1) In the resting state the intracellular concentration of ionized (free) calcium is within the range 0.05-0.5 μM whereas that of the extracellular fluid is 1-10 mM. On electrical or chemical stimulation, the intracellular free calcium is increased by one to two orders of magnitude. (2) There exist within the cell specific calcium-binding proteins with dissociation constants for calcium of between 10^{-7} and 10^{-5} M and which serve as intracellular calcium receptors. (3) Within the plasma membrane and intracellular organelles, calcium-specific entry, exit, release, and uptake processes exist. These processes function both to generate the elevated levels of calcium during excitation and to restore and maintain the low intracellular free calcium levels of the resting state.

Calcium Distribution.

Cellular calcium is distributed among several extracellular and intracellular compartments. Most cells are surrounded by an extracellular coat of proteins and mucopolysaccharides

called the glycocalyx, which can bind large amounts of calcium (Chambers, 1940; Burgos, 1960; Brandt, 1962; Gasic & Gasic, 1962; Bennet, 1963; Cook et al., 1965; Borle, 1968; Howell & Tyhurst, 1976). Calcium is also bound to the phospholipids and to the structural proteins of the cell plasma membrane (Tobias et al, 1962; Carvalho et al., 1963; Koketsu et al., 1964; Palmer et al., 1970; Quinn & Dawson, 1972; Duffy & Schwarz, 1973). 50-90% of the cell calcium is bound extracellularly depending on the tissue (Borle, 1968; van Rossum, 1970; Claret-Berthon et al., 1977; Muriarty, 1977; Tupper & Zorgniotti, 1977). Within the cells, the intracellular calcium storage pools contain widely different amounts of calcium: the cytosol, the nucleus, the mitochondria, the endoplasmic or sarcoplasmic reticulum, inner aspect of the plasma membrane, and possibly secretory vesicles. Mitochondria contain 3 times as much calcium as the microsomes derived from endoplasmic reticulum or sarcoplasmic reticulum. About 60% of the intracellular calcium is sequestered in these two subcellular organelles while only 10% is left in the supernatant or cytosolic fraction. 50% or less of the mitochondrial calcium is exchangeable. The unexchangeable fraction could possibly reflect the sequestration of calcium in mitochondria as calcium phosphate precipitates (Peachey, 1964; Greenawalt et al., 1964; Weinbach & von Brand, 1968; Zadunaisky et al., 1968; Lehninger,1970; Matthews et al., 1970; Martin & Matthews, 1970; Sutfin et al., 1971; Ruigrok & Elbers, 1972; Sayegh et al., 1974) or as organic-inorganic complexes (Barnard & Afzelius, 1972; Bonucci et al., 1973).

Importance of Cytosolic Ionized Calcium.

The cytosolic calcium activity is the most important aspect of the metabolism of cellular calcium. It is the ionized calcium that regulates the cell functions sensitive to calcium. The regulation of intracellular ionized calcium is clearly of central importance to cell function. It is indeed essential for cell viability. Calcium is not distributed passively across the plasma membrane. There is a large electrochemical gradient between the exterior and interior of the cell which makes the distribution of free calcium across the cell membrane far from equality but a proportionately much higher calcium concentration in extracellular fluids. The maintenance of this uneven distribution indicates the existence of specific active mechanisms for the extrusion and sequestration of intracellular calcium to maintain intracellular calcium homeostasis.

Calcium Withdrawal and Its Storage.

Several mechanisms for the active transport of calcium out of the cell, across the plasma membrane, have been described; (1) an ATP-dependent calcium efflux mediated by a calcium-sensitive, magnesium-dependent ATPase (CaMgATPase) that derives its energy from the hydrolysis of ATP; (2) an efflux of calcium in exchange for extracellular sodium ions (Na-Ca exchange) that derives its energy from the sodium electrochemical gradient established across the plasma membrane by the enzyme Na-K-ATPase; (3) an efflux of calcium

in exchange for an extracellular calcium ion (Ca-Ca exchange) that derives its energy from the calcium electrochemical gradient.

ATP-dependent calcium efflux and its relation to the enzyme CaMgATPase have been extensively studied in erythrocytes, where the only possible controller of the intracellular free calcium concentration is the ATP-dependent calcium transport across the plasma membrane, which is catalyzed by CaMgATPase. However, most other cells of tissues, for example, kidney (Parkinson & Radde,1971; Kinne-Saffran & Kinne,1974; Moore et al., 1974; Gmaj et al., 1979), intestine (Kurebe, 1978, 1979; Ghijsen & van Os, 1979), brain (Ohashi et al., 1970; Blitz et al., 1977; Rahamimoff & Abramovitz, 1978), nerves (DiPolo, 1976; Baker, 1976), skeletal muscle (Sulakhe et al., 1973; Casteels et al., 1973), smooth muscle (Thorens, 1979), exhibit some kind of ATP-dependent calcium transport across their plasma membrane or some CaMgATPase activity. The enzyme is inhibited by lanthanum, ethacrynic acid, mersalyl, chlorpromazine and ruthenium red; it is not inhibited by ouabain, oligomycin, sodium azide, fluoride or caffeine (Schatzmann & Vincenzi,1969; Vincenzi, 1971; Weiner & Lee, 1972; Schatzmann, 1975; Quist & Roufogalis, 1975; Hinds et al., 1978). Many investigators have found that an activator protein called calmodulin stimulates the CaMgATPase activity from two- to fourfold. Calmodulin also acts as the calcium-dependent regulator of a host of biochemical and physiological events. Low- and a high-affinity binding sites of CaMgATPase for calcium have been detected (Schatzmann &

Rossi, 1971; Quist & Roufogalis, 1975, 1977; Gopinath & Vincenzi, 1977; Scharf & Foder, 1978). Scharf and Foder (1978) presented evidence that the enzyme exists in two different and reversible states: (a) a resting state with low affinity for calcium (Kca=30 μM), a Hill coefficient of 1, a low maximum velocity, and (b) an active state with high affinity for calcium (Kca=1 μM), a Hill coefficient greater than 1, suggesting a positive cooperativity of calcium activation, and a high maximum velocity. The resting state corresponds to the enzyme free of the activator protein calmodulin, whereas the activator would be bound to the enzyme in the active state. Scharf and Foder (1978) also showed that the shift from resting to active state occurs when the enzyme is exposed to increasing calcium concentrations in the presence of calmodulin. It has been found that calmodulin is not the only activator of CaMgATPase, the ATPase purified from the erythrocyte membrane can also be stimulated by a number of acidic phospholipids, by polyunsaturated fatty acid, and by a limited proteolytic treatment.

In 1958, Luttgau and Niedergerke (1958) reported that frog hearts depolarized in high-KCl media can be made to contract or to relax, respectively, by reducing or increasing the proportion of Na in the surrounding fluids. Reuter and Seitz (1968) were the first to suggest an exchange diffusion countertransport by which calcium could be transported out of the cells in exchange for Na against a large electrochemical gradient without direct metabolic energy coupling.

An extracellular calcium-dependent calcium efflux has been reported by many investigators (Blaustein & Hodgkin, 1969; DiPolo, 1974; Blaustein, 1974, 1977; Blaustein et al., 1974; Blaustein & Russell, 1975; Baker & McNaughton, 1976, 1978; Baker, 1978). Since calcium influx is also stimulated by raising intracellular calcium (Blaustein & Russell, 1975; DiPolo, 1979), Blaustein proposed that this represents a Ca-Ca exchange mediated by the same carrier as the Na-dependent calcium efflux, sodium and calcium competing for the same carrier site.

The uptake of intracellular free calcium by intracellular calcium stores is an important factor that maintains intracellular calcium homeostasis with the exception of erythrocytes. Calcium pumps exist in sarcoplasmic reticulum, endoplasmic reticulum, mitochondria, and plasma membrane. Although the affinities for calcium, Kca, are not very different, the velocity of the plasma membrane transport system is smaller than in microsomes and several orders of magnitude less than in mitochondria and sarcoplasmic reticulum, suggesting that calcium transport across the membrane may be comparatively ill-suited to control the "set point" of the cytosolic calcium activity. Since the mitochondrial inhibitor azide causes a rapid rise in intracellular free calcium it is likely that mitochondria play an important role as a controller of cytosolic calcium (Simonsen & Christofferson,1979). The cytosolic exchangeable calcium pool changes little when the extracellular calcium concentration is altered between 0.7 and 2.5 mM, most of the

gain or loss in cell calcium is seen in the mitochondrial calcium pool.

In fact more than 40% of the total cell calcium is stored in mitochondria, constituting the largest calcium compartment of the cell. The mitochondrial calcium transport is explained on chemiosmotic theory. During equilibrium-state oxidative phosphorylation the proton electrochemical potential difference ($\Delta\mu_{H}+$) generated by proton extrusion linked to the respiratory chain is utilized to drive a proton-translocating ATPase in reverse, so that a net synthesis of ATP occurs. The proton electrochemical potential difference has both electrical and concentrative components, and the extrusion of H^{+} results in the buildup of a membrane potential (positive outside the matrix) and a pH gradient (acid outside the matrix).

$$\Delta\mu_{H}+=\Delta\psi-60\Delta pH$$

where $\Delta\psi$ is the membrane potential (positive when the matrix is negative) and ΔpH is the transmembrane pH gradient (positive when the matrix is acidic). Calcium can be transported across the inner membrane by a uniport mechanism, i.e. as uncompensated calcium ion. The driving force for calcium accumulation is by definition the calcium electrochemical potential, given by:

$$\Delta\mu_{Ca}2+ =2\Delta\psi -60\log\frac{(Ca)\,matrix}{(Ca)\,medium}$$

Since $\Delta\psi$ is in the range 150-180 mV, it follows that the gradient of free calcium across the inner membrane could in theory attain values of 10^5-10^6 if this were the only means for transporting calcium across the inner membrane.

It has been proposed that there is a high-affinity calcium-binding site in mitochondria which reflects the active site of the carrier molecule with an affinity constant Kca of 0.025 μM (Reynafarje & Lehninger, 1969; Lehninger et al., 1978). Lanthanum is a competitive inhibitor of calcium transport into mitochondria, presumably competing with calcium for the same site on the carrier molecule. And indeed several investigators have isolated from mitochondria a glycoprotein with affinity for calcium (Sottocasa et al., 1971, 1972; Gomez-Puyou et al., 1972; Kimura et al., 1972; Tashmukhamedov et al., 1972).

Some findings have indicated that except in erythrocytes and in skeletal and cardiac muscle fibers, mitochondria are the main controllers of the cytosolic calcium activity (Lehninger, 1964; Bygrave,1967, 1978; Borle, 1973; Mela, 1977; Carafoli & Crompton, 1978b). However, there are objections against the major role of mitochondria in controlling cytosolic free calcium: (a) the affinity of mitochondria for calcium is simply too low (Kca>1 μM); (b) calcium cycling across the inner mitochondrial membrane appears to be negligible at the physiologic cytosolic free calcium concentration ($0.5\text{-}3.0\times10^{-7}$ M) (Scarpa, 1975; Scarpa et al., 1978; Brinley, 1978; Brinley et al., 1978). Several lines of evidence suggest that mitochondria do play a

important role in cellular calcium metabolism. First, the major part of the cell calcium is found in mitochondria. Second, when a cell is taking up calcium (during calcium loading or in tracer experiments) the major part of the load is taken up by the mitochondria in liver, kidney, muscle, salivary glands, synaptosomes and in the squid axon. Third, Rose and Lowenstein (1975) showed that intracellular microinjections of calcium in the giant salivary gland cells of chironomus were rapidly buffered by an energy-dependent process and did not increase the cytosolic ionized calcium monitored with aequorin. However, when cyanide or ruthenium red were injected before calcium, there was a large and diffuse increase in aequorin luminescence, indicating a large rise in cytosolic free calcium. Since ruthenium red inhibits mitochondrial calcium uptake without affecting calcium transport by microsomes or by the plasma membrane (Ash & Bygrave, 1977; Blaustein et al., 1978), these experiments suggest that mitochondria are the principal controllers of cytosolic free calcium in these salivary gland giant cells. In fact, the mitochondrial calcium transport system has high maximal velocity and capacity to sequester calcium. In spite of a Kca that is one order of magnitude larger than the cytosolic calcium activity the rate of calcium transport of mitochondria is so high that even at physiologically low calcium concentrations, its transport capacity or rate of cycling is significant and predominates over the plasma membrane calcium transport system. Another important consideration is the relative mass and the relative surface area of mitochodria transport

system. Work by Weibel et al. (1969) showed that the surface of the inner mitochondrial membrane was 21 times greater than the plasma membrane surface in the rat liver. Furthermore, the mitochondrial calcium uptake increases more than that of plasma membrane with increasing cytosolic free calcium. Experiments have shown that in liver, kidney, adipocytes, nerve terminals, and smooth muscle the rate of calcium uptake and the capacity of calcium sequestration of mitochondria exceed by least one order of magnitude those of endoplasmic reticulum and of plasma membrane (Carafoli & Tiozzo, 1967; Batra & Daniel, 1971; Batra 1973, 1974; Moor et al., 1974; Wikstrom et al., 1975; Vallieres et al., 1975; Bruns et al., 1976; Janis et al., 1977; Malmstrom & Califoli, 1977; Ash & Bygrave, 1977; Farber et al., 1977; Bygrave & Trauter, 1978). Therefore, the importance of mitochondria in controlling intracellular calcium homeostasis in these tissues must be considered.

In skeletal muscle the sarcoplasmic reticulum is recognized to be the principal controller of the cytosolic calcium. Detail of calcium transport in skeletal muscle has been studied using the reconstituted vesicles or microsomes from fragmented sarcoplasmic reticulum. In the muscle microsomes, calcium transport is stoichiometrically coupled to ATP hydrolysis: for each ATP hydrolyzed, 2 calcium atoms are carried across the membrane in a process that Mg-dependent. The active transport mechanism is the Mg-dependent Ca-activated ATPase that catalyzes the formation of a phosphoprotein intermediate. The sarcoplasmic reticulum affinity for calcium is in the same order of magnitude as

the Kca of the plasma membrane, endoplasmic reticulum and mitochondria. Its important kinetic property is the great velocity of the transport process reflected by a Vm that may exceed 1 μmol mg^{-1} prot. min^{-1}. Thus, it is accepted that skeletal muscle sarcoplasmic reticulum can lower the cytosolic calcium activity below 10^{-8} M.

In heart muscle, the sarcoplasmic reticulum is probably not the only controller of cytosolic calcium activity. The existence of calcium channels makes this tissue more dependent on the extracellular calcium concentration and on calcium influx and efflux through the plasma membrane. In addition, many investigators have suggested that mitochondria may play a role in the control of free calcium in heart muscle (Chance, 1965; Patriarca & Carafoli, 1968; Harigaya & Schwartz, 1969; Carafoli & Azzi, 1972; Carafoli et al., 1972; Lehninger, 1974; Affolter et al., 1976; Carafoli & Crompton, 1978a; Lentz et al., 1978; Nayler et al., 1979). Others doubt it because of the mitochondrial high Kca and their slow transport velocity compared with that in sarcoplasmic reticulum (Solaro, 1972; Scarpa & Graziotti, 1973; Scarpa, 1975; Tsokos et al., 1977). Tsokos et al. (1977) suggested that, although mitochondria may not play a significant role in the rapid uptake of calcium promoting relaxation of myofibrils, the possibility still remains that they participate in regulating the steady-state calcium levels and the resting tension of the myofibrils.

Calcium Sources and Mobilization

There may be as many as three different sources of calcium

for contraction: (1) extracellular calcium,(2) Calcium bound to receptors,and (3) calcium released from intracellular calcium stores, which are present on the inner surface of cell membranes, in mitochondria and endoplasmic or sarcoplasmic reticulum.

The entry of the extracellular calcium can be (a) by passive diffusion down the concentration gradient, (b) in exchange for internal sodium or other ions and (c) operated by two types of ion channel in the cell membrane: the voltage-dependent calcium channel (VDC) and the receptor-operated calcium channel (ROC). The voltage-dependent channels have been defined as those activated by membrane depolarization (electrical or high potassium), while the receptor-operated channels are those associated with membrane receptors and are activated by specific agonist-receptor interaction. It is not known if the channels themselves are different structures or if the association of ligand receptors with voltage-dependent channels changes their voltage dependence and sensitivity to channel antagonists and make them receptor-operated channels. In any smooth muscle several different stimulants may have their own receptors, each receptor type operating its own ion channels with their particular ionic selectivity. It is easy to appreciate how the maximal contractile responses to various stimulants may not be the same, because the increase in intracellular free calcium concentration produced by different stimulants is unequal.

The process of release of calcium from intracellular stores

(mitochondria and sarcoplasmic or endoplasmic reticulum) needs to be triggered by calcium from other sources. Trigger calcium may come from the extracellular source either through voltage-dependent ion channels or through ROCs. It may also be dislodged from a bound site associated with the receptor. At low concentrations of stimulants the action potential (AP) is the important source of trigger calcium in cells that generate APs. In cells that do not readily generate APs, calcium entering through ROCs is the main source of trigger calcium, and this source is also quantitatively the most important in cells that normally generate APs when high concentrations of stimulants are applied and AP discharge is abolished. A voltage-dependent opening of calcium ion channels in smooth muscle cells not normally generating APs may be a minor source of trigger calcium when these are depolarized by stimulant drug application. The relative extent of intracellular and extracellular sources is dependent on the tissue, stimulant, species, the environment of the calcium channels, and the effect of other calcium regulating mechanisms (Bolton, 1979; Casteels, 1980; Danniel, Crankshaw & Kwan, 1979; Putney, 1978; van Breemen, Aaronson, Loutzenhiser & Meisheri, 1980).

Calcium Channel Blockers

The ubiquitous role of calcium in cell regulation and the diversity of processes controlling cellular calcium concentration indicate the importance of identification of the sources and routes of calcium mobilization. Several

approaches have been used to examine fundamental processes of calcium ion metabolism at the cellular level and to estimate the relative importance of different calcium stores for the mechanism of activation. One approach is to use agents that selectively antagonize the pathways of calcium utilization. Considerable attention has been paid in recent years to two major groups of compounds ---- the calmodulin antagonists and the calcium channel blockers.

The description of calcium channel blockers owes much to the original investigations of Fleckenstein who first observed that verapamil mimics the cardiac effects of calcium withdrawal (Fleckenstein, 1964). Since the original studies with verapamil, a large number of additional structures have joined this class of calcium channel blockers.

The calcium channel blockers are a structurally and pharmacologically heterogeneous group of drugs. The obvious structural heterogeneity suggests that more than one site and mechanism of action exist. The inorganic cations Mn^{2+}, Co^{2+} and La^{3+} apparently act as general antagonists and are effective in a wide variety of calcium-dependent processes (Bolton, 1979; Mikkelsen, 1976; Parod & Putney, 1980; Rosenberger, Ticku & Triggle, 1979; Triggle & Swamy, 1980). This nonselectivity of action probably arises from a general ability of these cations to substitute for calcium at calcium-binding sites. It is possible that these cations occupy the cation coordination site of calcium channels and either block channel function or actually enter the cell (Fukuda & Kawa, 1977; Hagiwara, 1975), where they can

substitute for or block calcium binding at the intracellular calcium receptors.

The organic calcium channel blockers are generally competitive with calcium, but it is clear that they lack the widespread calcium-blocking properties of the inorganic cations and important differences in activity can be seen among the various structural classes of these compounds. They are grossly divided into three classes in terms of the binding of the labelled calcium antagonist to the calcium channel. Those which compete according to the law of mass action for an interaction at one binding site (Hill slope=1.Ø), are designated Class I, to which all of the 1,4-dihydropyridines, for example, nifedipine and nimodipine, belong. Those which compete in a more complex manner, suggesting interaction at multiple sites (Hill slope<1.Ø), are termed as Class II and include verapamil and D 6ØØ (the methoxy derivative of verapamil). There is a third class of calcium channel blocker, of which diltiazem is representative. It stimulates the binding of tritium-labelled nimodipine by decreasing the Kd value.

Class II drugs, verapamil and D 6ØØ, are the most intensively studied organic calcium blockers. The specific mechanism of action of these drugs in blocking calcium entry is known. Extracellular calcium can enter the cell by passive diffusion, ion exchange and calcium channels, as mentioned above. Only the pathway which involves the inward movement of calcium through the voltage-dependent channels has been shown to be sensitive to verapamil (Fleckenstein,

1977; Kohlhardt, Bauer. Krause & Fleckenstein, 1972). For this reason, the term "slow channel blocker" more accurately describes their mode of action; the term "slow" refers to the relatively slow rate of opening of these channels compared with that of the channels which are responsible for the influx of sodium during the rapid depolarization phase of the action potential in the heart. The slow channels also admit some sodium ions, and verapamil and D 600 inhibit the sodium as well as the calcium transporting capacities of these channels (Kass & Tsien, 1975). It is now known that high concentrations of verapamil affect the fast inward sodium current (Bayer, Kalusche, Kaufmann & Mannhold, 1975).

In addition to the blockade of the slow inward calcium current in cardiac tissue, they serve as competitive antagonists against extracellular calcium in smooth muscle and are potent inhibitors of smooth muscle contractile activity produced by potassium depolarization (Hashimoto, Nakagawa, Nabata & Imai, 1978; Imai, 1979; Reimer, Dorfler, Mayer & Ulbrecht, 1974). They are also potent antagonists of some agonist-induced mechanical events, and there is a reasonably good correlation between this activity and the dependence of the response on extracellular calcium (Farley & Miles, 1978; Harris, Swamy, Triggle & Waters, 1980; Jetley & Weston, 1980; Rosenberger, Ticku & Triggle, 1979; Schumann, Gorlitz & Wagner, 1975; Shimizu, Ohta & Toda, 1980).

Stimulatory Actions of Calcium Antagonists and Possible Mechanisms

In this study it has been found that verapamil enhanced the contractile activity of prostanoids on the rat anococcygeus muscle. This is not due to release of noradrenaline by prostanoids, since the potentiating activity is not altered by sympathetic denervation with 6-hydroxydopamine or the presence of phentolamine. Several other stimulant actions of verapamil are known. On frog sartorius muscle verapamil produced contraction (Bondi, 1978). In rat diaphragm muscle verapamil increased the transmitter release from motor nerve terminals (Nishimura,Asai & Urakawa, 1982). Verapamil potentiated the response of the rat vas deferens to single pulse field stimulation (French & Scott, 1981). On rat kidney cortical slices verapamil stimulated the p-aminohippurate (PAH) uptake (Matsushima & Gemba, 1982). On the mouse leukemia cells, verapamil and diltiazem, but not nifedipine, causes a 3-fold enhancement of the frequency of transfer of the cloned gene for herpes simplex virus thymidine kinase (Akiyama, Ona & Kuwano, 1983).

It has been suggested that verapamil inhibits the membrane calcium pump and the associated activation of the calcium-sensitive ATPase (Mas-oliva & Nayler, 1980; Publicover & Duncan, 1979). In addition, Johnson et al. (1982), Bostrom et al. (1981) and Motlib, Vaghy, Johson and Schwartz (1982) showed that verapamil, D 600, diltiazem, felodipine and nisoldipine (nifedipine analogues) can bind to the hydrophobic binding sites of calmodulin. It is now

known that calmodulin plays a pivotal role in many cellular functions (Cheung, 1980). In the calcium-transport system, the CaMgATPase activity of erythrocytes is enhanced by calmodulin (Jarret & Penniston, 1977; Waisman, Gimble, Goodman & Rasmussen, 1981; Niggli, Penniston & Carafoli,1979). In addition to erythrocytes, calmodulin stimulates the calcium-transport system of the plasma membranes of most cell types, e.g. cardiac muscle (Caroni & Carafoli, 1981) and adipocytes (Pershadsingh, Landt & McDonald, 1980) by a direct interaction with CaMgATPase. It is proposed that verapamil might produce the potentiation by inhibiting CaMgATPase through binding to calmodulin.

Raess and Vincenzi (1980) reported that in the presence of calcium, antipsychotic drugs could bind to calmodulin and thereby inhibit calmodulin-stimlated CaMgATPase activity. This inhibition is related to the binding of the drugs to hydrophobic regions of calmodulin which are exposed by calcium-induced conformational changes. Phenothiazines and N-substituted-naphthalenesulfonamides have been widely used as tools to demonstrate the calmodulin-dependent process in isolated enzyme systems and at the level of intact cells (Weiss, Prozialeck, Cimino, Barnette & Wallace, 1980; Nishikawa, Tanaka & Hidaka, 1980).

A number of calmodulin antagonists were then tested in this study. Most of the phenothiazines tested and propranolol produced enhancement of the action of prostanoids on the rat anococcygeus muscle preparation. Since CaMgATPase can also be stimulated by a limited

proteolytic treatment, trypsin was tested. It inhibited the response to 11,9-epoxymethano PGH2, which appeared to support the idea that the membrane CaMgATPase played a important role in regulating intracellular free calcium in the rat anococcygeus muscle. For these reasons, it was assumed that those calcium antagonists produced their enhancement by binding to calmodulin and preventing CaMgATPase from pumping calcium out of the cell. Thus, intracellular free calcium which is essential for contraction would be increased. These findings seemed to support our above assumption.

However there is evidence against this hypothesis. The order of pP2 values for the calmodulin antagonists differs from the Ki values for the displacement of tritiated-trifluoperazine from calmodulin (Weiss, Prozialeck, Cimino, Barnette & Wallace, 1980). Pimozide, which is the most potent compound in displacing tritiated-trifluoperazine produced no enhancement on the rat anococcygeus muscle preparation. Tetracaine, a local anesthetic, inhibits the specific calmodulin-dependent stimulation of erythrocyte CaMgATPase. However, xylocaine, which is a compound structurally similar to tetracaine, inhibited the response to 11,9-epoxymethano PGH2. Nifedipine, a structural analogue of felodipine has been shown to have little potentiating effect (Bostrom et al., 1981). Furthermore, Epstein, Fiss, Hachisu and Andrenyak (1982) showed that verapamil was a weak inhibitor against calmodulin-sensitive forms of cyclic AMP phosphodiesterase. And verapamil does not antagonize the calcium stimulation of

aortic myosin light chain kinase or ATPase, which are presumed to be mediated through calmodulin (Kanamori, Naka, Asano & Hidaka, 1981).

CaMgATPase can also be stimulated by acidic phospholipids and polyunsaturated fatty acids (Niggli, Adunyah, Penniston & Carafoli, 1981; Niggli, Adunyah & Carafoli, 1981; Stieger & Schatzmann, 1981). It has been reported that calmodulin antagonists can interact not only with calmodulin but also with phospholipids and inhibit phospholipid-stimulated phosphodiesterase (Kanno & Sasaki, 1982; Adunyah, Niggli & Carafoli, 1982), and calmodulin antagonists may even have a direct interaction with the CaMgATPase (Vincenzi, Adunyah, Niggli & Carafoli, 1982). However, at low temperature (12°C), where CaMgATPase will completely lose its activity (Duncan, 1976), verapamil and chlorpromazine were still capable of potentiating responses to prostanoids, although higher concentrations were needed.

It is suggested that verapamil also blocks sodium channels (Shigenobu, Scheider & Sperelakis, 1974). The increase in the sodium gradient and stimulation of NaK-ATPase activity can not explain the stimulatory action of verapamil, since in the presence of ouabain the PAH accumulation was still stimulated by verapamil (Matsushima & Gemba, 1982).

Caffeine in high concentrations produces smooth muscle contraction. Its mechanism is not known. Kurebayashi & Ogawa (1981) reported that the amount of calcium release by chlorpromazine from the loaded fragmented sarcoplasmic reticulum of bullfrog skeletal muscle was increased by a

rise in calcium concentration and decreased by procaine or an increase in magnesium concentration, and that chlorpromazine may act in a way similar to caffeine. However their further experiments showed chlorpromazine evoked tension by a different mechanism: calcium release by chlorpromazine from fragmented sarcoplasmic reticulum loaded with calcium in the presence of carbamylphosphate was markedly decreased during the initial phase; in the presence of carbamylphosphate, β-Y-methylene adenosine triphosphate, a nonhydrolyzable analogue of ATP, does not potentiate the calcium releasing action of chlorpromazine. These are in contrast to the action of caffeine. And the action of chlorpromazine in the presence of caffeine was difficult to explain by the assumption that they might act on the same site. Other workers have shown verapamil elicited contraction through a different way from caffeine. Elimination of extracellular calcium partially depressed verapamil-induced contraction without significantly blocking caffeine-induced contraction and sarcoplasmic reticulum calcium release (Frank, 1962; Luttgau, 1963; Putney & Bianchi, 1974). And caffeine-induced contraction is totally inhibited by procaine while verapamil induced contraction is only partially inhibited by procaine (Bondi, 1978).

These unsatisfactory explanations urged us to look for other possibilities. It is known that phenothiazines possess some unusual properties. Caroni & Carafoli (1981) reported that phenothiazines caused leakage of the plasma membrane of cardiac muscles. N-(6-aminohexyl)-5-chloronaphthalene-1-sulfonamide (W-7), trifluprazine and

trifluoperazine induced renin release from isolated glomeruli in a dose-dependent manner (Kawamura & Inagami, 1983). Naccache, Molski, Alobaidi, Becker, Showell and Sha'afi (1980) reported that trifluoperazine induced the release of lactate dehydrogenase from rabbit peritoneal neutrophils. In saponin-treated guinea pig peritoneal macrophages, chlorpromazine, trifluoperazine, W-7 and N-(6-aminohexyl)-1- naphthalenesulfonamide inhibited calcium uptake and released accumulated calcium (Hirata, Suematsu & Koga, 1983). On fragmented sarcoplasmic reticulum from bullfrog skeletal muscle, chlorpromazine caused calcium release and inhibited calcium uptake (Kurebayashi & Ogawa, 1981). Hirata, Suematsu and Koga (1982) found that addition of 100 μM phenothiazines caused leakage of the endoplasmic reticulum membrane and released more than 50% of the calcium taken up by the endoplasmic reticulum. Trifluoperazine inhibited calcium uptake and produced sudden release of calcium from sarcoplasmic reticulum vesicles of rabbit skeletal muscle, and inhibited calcium accumulation by rat cardiac sarcoplasmic reticulum in situ (Ho, Scales & Inesi, 1983). Sulfhydryl agents can release calcium from isolated rabbit sarcoplasmic reticulum vesicles, and chlorpromazine acts synergistically with the sulfhydryl agents (Bindoli & Fleischer, 1983). Chlorpromazine was found to make the contractile system more sensitive to calcium ion in fast twitch smooth muscle in a skinned fiber preparation and the main site of action was interpreted to be on the calcium release mechanism (Meissner & Fleischer, 1971).

Verapamil and D 600 showed similar properties. They caused

calcium release from frog sartorius muscle (Bondi, 1978). At the frog neuromuscular junction, verapamil and D 600 at high concentration caused the release of calcium from intracellular calcium stores and raised intracellular free calcium (Publicover & Ducan, 1979), and they suggested that verapamil generates tension by a mechanism other than depolarization. Furthermore, verapamil inhibits calcium binding in preparations of isolated cardiac sarcoplasmic reticulum (Nayler & Szeto, 1972; Watanabe & Besch, 1974; Mas-Oliva & Nayler,1980) and calcium uptake by isolated rabbit skeletal muscle sarcoplasmic reticulum (Balzer, 1972). Pang and Sperelakis (1983) have proved that verapamil enters and accumulates in the cardiac and smooth muscles, and internal verapamil concentration at steady state for both cardiac and smooth muscle is much higher than the drug concentration in the medium. Nayler, Mas-Oliva & Williams (1979) have shown verapamil binds to isolated rabbit cardiac sarcolemmal fragments and particularly to carbohydrate-containing residues. Lullmann, Timmermans & Ziegler (1979) have reported that verapamil was accumulated by isolated left atria of the guinea pig. The uptake was enhanced by electrical stimulation of the muscle. The accumulation of verapamil by cardiac tissue has also been confirmed by Keefe and Kates (1982), who found a myocardial/plasma concentration ratio of 6.2 following intravenous administration of verapamil into anesthetized dogs. These facts indicate that those drugs may produce their stimulatory action by acting on intracellular calcium stores ---- inhibiting calcium uptake by and releasing

calcium from sarcoplasmic reticulum and mitochondria.

The release of calcium from sarcoplasmic reticulum can be triggered by calcium from other sources or by nerve impulses which propagated into the sarcoplasmic reticulum. This calcium uptake into the sarcoplasmic reticulum is an ATP-dependent process representing the operation of a CaMgATPase located in the membrane. Calmodulin has been reported to stimulate calcium transport in the sarcoplasmic reticulum of cardiac muscle (Katz & Remtulla, 1978; LePeuch, Haiech & Demaille, 1979), and calmodulin has been found in endoplasmic reticulum of both lobster muscle and rat liver (Carafoli, Niggli, Malstrom & Caroni, 1980). Therefore, it was considered that calcium antagonists might bind to calmodulin in sarcoplasmic reticulum and inhibit CaMgATPase, so that the intracellular free calcium would be increased. However, Pershadsingh, Landt & McDonald (1980) found that, although calmodulin stimulated calcium transport in plasma membrane vesicles of adipocytes prepared from rat epididymal fat pads, it did not stimulate calcium transport in endoplasmic reticulum-enriched adipocyte microsomes. So, it appears that the calmodulin stimulation of calcium transport occurs in plasma membranes but probably not in endoplasmic reticulum, and that the sarcoplasmic reticulum of cardiac muscle was an exception, perhaps due to the existence of a special protein, phospholamban, in cardiac sarcoplasmic reticulum (LePeuch, Haiech & Demaille, 1979). Thus, whether calmodulin would stimulate calcium transport in the cytoplasm is controversial. Recently, Hirata, Suematsu and Koga (1983) obtained new evidence supporting the idea that

calmodulin does not enhance either the maximal capacity for calcium uptake or the apparent affinity for calcium of calcium accumulation by the endoplasmic reticulum of guinea pig peritoneal macrophages, under the condition of saponin treatment in the presence of sodium azide, an inhibitor of mitochondrial calcium uptake. They also discovered that phenothiazine inhibition of calcium uptake required a higher concentration than did inhibition of calmodulin stimulation of phosphodiesterase activity (Levin & Weiss, 1977; Raess & Vincenzi, 1980). The order of potency of inhibition of phosphodiesterase activity by various calmodulin antagonists was not applicable to inhibition of calcium uptake. Furthermore, inhibition of calcium uptake by phenothiazines was not reduced by the addition of excess calmodulin (Shenolikar, Cohen, Cohen, Nairn & Perry, 1979). And the inhibition of calcium uptake by calmodulin antagonists was not modified by changes in the concentration of either Mg-ATP or free calcium , both of which are important factors in calcium uptake. Therefore, the inhibitory effect on calcium uptake by calmodulin antagonists does not seem to be due to a specific effect on endogenous calmodulin.

Another possibility is that these drugs inhibit calcium uptake of mitochondria or enhance the calcium release. Hirata, Suematsu and Koga (1982) demonstrated that phenothiazines and N-substituted-naphthalenesulfonamides at low concentration had an inhibitory effect on the calcium uptake of the mitochondria of the guinea-pig macrophage. This inhibition is probably not due to a specific antagonist

effect of these drugs on calmodulin: (1) exogenously added calmodulin did not affect calcium uptake by the mitochondria, in terms of both the capacity and the apparent affinity for calcium, (2) exogenously added calmodulin did not affect the inhibitory effect of phenothiazines, (3) almost complete inhibition by phenothiazines and N-naphthalenesulfonamides occurred; if the inhibition of calcium uptake by these drugs is due to specific effects on calmodulin, all of the mitochondrial calcium uptake would be dependent on the activation of calmodulin. Furthermore. Carafoli, Niggli, Malmstrom & Caroni (1980) reported that calmodulin was either absent in liver mitochondria, or present only in negligible amounts, and the mitochondrial calcium uptake of liver and heart was not affected by the exogenous addition of calmodulin. Both verapamil (Appel, 1962) and phenothiazines (Carey, Hirom & Small, 1976) are lipophilic substances and at appropriate pH they will become cationic detergents to produce surface activity and disturb the ordered phospholipid bilayer structure of membrane. It has been proved that phenothiazines preferentially bind to phosphatidylserine on the inner half of the lipid bilayer (Carey, Hirom & Small, 1976). Therefore, calcium antagonists may produce their inhibition of calcium uptake and release of calcium by their detergent-like activity.

Since the proton efflux in the inner membrane of mitochondria supplies the energy for mitochondrial calcium uptake, as mentioned before, any drugs which can affect mitochondrial metabolism or membrane permeability will change calcium release from or uptake into mitochondria. The

following studies of the effect of chlorpromazine on mitochondria with respects to respiratory activity and potassium metabolism may help to explain the mechanism for the potentiating effect produced by calcium antagonists. Chlorpromazine inhibits respiratory activity by acting on different substrates, causes membrane de-energization (Byczkowski & Korolkiewicz, 1978; Byczkowski, 1977; Chazotte & Vanderkooi, 1981), and increases the mitochondrial membrane passive permeability to monovalent cations (Byczkowzki & Borysewicz, 1976, 1979; Byczkowzki, 1983). Depending on the external potassium concentration, chlorpromazine caused the passive shrinking or swelling of mitochondria due to efflux of potassium from their matrix in hypopotassium medium or influx of potassium into the matrix in hyperpotassium medium. It is generally believed that prior to transport into the mitochondrion, potassium cations bind temporarily to fixed superficial anionic sites such as phospholipids which, in addition, may serve as cation exchangers with the inner mitochondrial membrane (Byczkowski, Salamon, Harlos & Porter, 1981). Chlorpromazine (0.25 mM) produced partial neutralization of the surface potential of intact mitochondria. Byczkowzki (1983) postulated this neutralization may be caused by electrostatic interaction of the nitrogen atom of chlorpromazine molecule, positively charged at pH 7.2, with negatively charged binding sites at the surface of inner mitochondrial membrane. Moreover, the lipophilic portion of chlorpromazine molecule is able to interact with lipid phase of the membrane (Byczkowski, 1977) and even form a

charge-transfer complex (Szent-Gyorgyi, 1960). As a consequence of electrostatic and lipophilic interaction of chlorpromazine with the inner mitochondrial membrane and subsequent dearrangement of the lipid phase the activation of endogenous permeability to osmotically active potassium occurred. Perhaps, both mechanisms are essential for chlorpromazine action, since surface potential neutralization alone by spermine or other polyamines (Byczkowski & Porter, 1981; Byckowski, Zychinski & Porter, 1982) caused no increase of mitochondrial membrane passive permeability of osmotically active cations, although the polyamines counteracted effects of chlorpromazine. On the other hand, purely lipophilic agents ---- derivatives of DDT, known for their interaction with lipid bilayer (Byczkowski, 1973, 1977), not only inhibited the carrier-mediated potassium transport in mitochondria (Chefurka, Zahradka & Bajura, 1980) but also reversed the effect of chlorpromazine (Byczkowski, 1977, 1978). More recently, Vale, Moreno and Carvalho (1983) have pointed out that the inhibitory effect of chlorpromazine and trifluoperazine on the respiration-dependent and ATP-dependent calcium uptake by rat liver mitochondria is due to direct action of the drugs on the electron carrier system of the respiratory chain. Therefore, it is possible that phenothiazines affect both potassium and calcium metabolism through the same mechanism ---- the inhibition of the respiratory chain.

In some tissues, e.g. rat uterine horn, PG-induced contraction requires extracellular calcium: the withdrawal of calcium fromthe bathing medium abolishes the contraction (Harbon, Vesin & Do Khac, 1975; Paton & Daniel, 1967). On the other hand, it has been reported that 11,9-epoxymethano PGH2 produces a submaximal contraction of rabbit aorta in the absence of extracellular calcium which is sustained for many hours, and it has been proposed the 11,9-epoxymethano PGH2-induced contraction is the result of mobilization of calcium from one of the intracellular calcium stores (van Breemen, Aaronson, Loutzenhiser & Meisheri, 1980; Loutzenhiser & van Breemen, 1981). Brandt, Andersson, Edvinsson and Ljunggren (1981) found that on human cerebral arteries potassium-contracted vessels relaxed almost completely, while PGF2a-contracted arteries were relaxed by only 60% when calcium antagonists were added. Shimizu, Ohta and Toda (1980) have also shown that in dog cerebral arteries, verapamil relaxed potassium-contracted arteries more then PGF2a-contracted vessels. Godfraind, Miller and Socrates Lima (1983) reported that the contraction induced by PGF2a in the rat aorta is partially dependent on intracellular calcium. In calcium-free solution, depolarization with 100 mM potassium failed to produce a contraction of rat aorta, but PGF2a (3 μM) stimulated a contraction equal to about 23% of maximal elicited in normal physiological solution. More recently Heaslip & Rahwan (1982) have demonstrated that in rat aorta

the persistent contraction induced by 11,9-epoxymethano PGH2 under calcium-free conditions is not diminished by nifedipine, but is blocked by 2-n-butyl-3-dimethylamino-5,6-methylenedioxyindene (bu-MDI). bu-MDI is a tertiary amine with predominantly intracellular calcium antagonistic properties (Rahwan & Witiak, 1979; Rahwan, Faust & Witiak, 1977; Rahwan, Witiak & Muir, 1981; Rahwan & Gerald, 1981; Piascik, Johnson, Potter & Rahwan, 1981; Burchfield & Rall, 1981). In further support of this contention is the finding by Heaslip & Rahwan (1982) that quaternary-bu-MDI, which in all likelihood has very limited access to the intracelllular compartment, is incapable of diminishing the contractile response of the aorta to 11,9-epoxymethano PGH2 elicited in the absence of extracellular calcium. In addition, Grosset & Mironneau (1977) have shown in uterine smooth muscle from pregnant rats that PGE1 increase the late outward current; it has been suggested in several other preparations that the increase in the intracellular calcium concentration could, in turn, lead to an increase in the outward current (Meech & Standen, 1975; Bassingthwaighte, Fry & Mcguigan, 1976). Furthermore, it has been shown in several studies that PGs may release calcium ions from isolated sarcoplasmic reticulum (Carsten, 1973) and mitochondria (Kirtland & Baum, 1972; Malmstrom & Carafoli, 1975; Tsuyoshi & Develin, 1979). These results may be interpreted most easily by assuming that prostanoids induce the release of calcium ions from intracellular storage pools into the cytoplasm as well as admit extracellular calcium ions.

In the present study, the alteration of extracellular

calcium concentration over the range 0.6-5.0 mM did not affect 11,9-epoxymethano PGH2 activity on the rat anococcygeus muscle preparation. However, in the calcium-free solution the maximum response to 11,9-epoxymethano PGH2 was markedly reduced. This seems to accord with the above observations that PGs can not only manoeuvre extracellular calcium but also intracellular calcium. However, verapamil and D 600 enhanced the actions of PGs instead of depressing them, even in the absence of calcium in the bathing medium. This raises a question: Do PGs really require extracellular calcium for contraction in this preparation? If so, why do calcium channel blockers fail to block PGs action? if not, what role does the extracellular calcium play in PG-induced contraction? Possible assumptions could be that (1) interaction of PGs with their receptors needs extracellular calcium, (2) extracellular calcium may help PG transport into the cell, if PG receptors located inside the cell, or (3) PGs release membrane-bound calcium, which comes from an extracellular source, to trigger further release of calcium from intracellular calcium stores.

From a search of the literature, only one example of a similar potentiating effect has been found. D 600 potentiates the contractile response to PGE2 in the uterine smooth muscle of pregnant rats (Grosset & Mironneau, 1977). In another group of experiments, Reiner & Marshall (1976) showed D 600 neither enhanced nor inhibited PGF2a-induced tonic contraction in the uterine smooth muscle of pregnant rats, but it is noteworthy that in their study a high

concentration of PGF2a (1Ø μM) was used. It is possible that this was a supramaximum concentration and no further increase in tension was attainable.

Receptor-response coupling can be altered (1) by compounds interacting with membrane receptors, (2) by action on the chain of events between the binding of an agonist to its receptor and the activation of actomyosin-ATPase which follows, or (3) by acting on the effectors, i.e. muscle filaments. Calcium antagonists may produce their enhancement by acting on any of these points.

Obviously, calcium antagonists give the enhancement not by interacting with the effectors, since calcium antagonists do not potentiate contractions due to high potassium or noradrenaline. With high potassium, one may argue that high potassium-induced contraction requires extracellular calcium and calcium antagonists block the channels, so the enhancement can not be seen. But in the absence of calcium in the bath solution, noradrenaline was able to cause contraction and the contraction was not blocked by verapamil, suggesting the release of intracellular calcium by noradrenaline.

Drugs may increase the level of cytoplasmic free calcium by enhancing the influx of extracellular calcium, by mobilizing intracellular calcium, or by preventing sequestration or efflux of cytosolic calcium. It is likely that calcium antagonists act on intracellular calcium stores, particularly mitochondria, since the mitochondrion in smooth muscle is an important regulator and calcium antagonists are

capable of releasing calcium from it.

Now the question is where and how PGs act. One could assume that (1) PGs can act directly on mitochondria by binding to their receptors in/on mitochondria to release calcium, or (2) PGs release calcium from mitochondria either by means of "trigger calcium" which is released by PGs from other calcium sources, or by means of other second messengers.

Our results suggest that contraction induced by PGs in the rat anococcygeus muscle is a receptor-mediated process, since (1) PG-induced contraction is antagonized by specific receptor blockers or partial agonists, (2) the order of potency for ICI 80205, 16,16-dimethyl PGE2 and PGE2 to enhance contraction by 11,9-epoxymethano PGH2 in rat anococcygeus muscle is the same as that for their contractile activities on other preparations, and (3) the order of potency of several TxA analogues is similar to that in other smooth muscle preparations. But these findings may not fit in with an action on intracellular organelles.

Several investigators have tried to prove PGs can bind to and release calcium from mitochondria (Kirtland & Baum, 1972; Malmstrom & Carafoli, 1975; Tsuyochi & Develin, 1979; McNamara, Roulet, Gruetter, Hyman & Kadowitz, 1980). Malmstrom and Carafoli (1975) pointed out that the concentrations of PG necessary to demonstrate calcium release from liver mitochondria are much greater than those required for pharmacological actions; they concluded the PG-induced liver mitochondrial calcium release did not reflect a physiological situation. On the other hand,

Somlyo, Somlyo, Devine, Peters & Hall (1974) found that 39% of the mitochondria in rabbit mesenteric vein are within 100 nm of the cell membrane. McNamara et al. (1980) assumed there was a relative high concentration of PGs in the vicinity of the mitochondria. At these high concentrations the surface active properties of PGs may affect the mitochondria. It has been shown that PG molecules will aggregate at an interface and will reduce the surface tension of water at 10 µg/ml. Since micelle formation can not be detected, orderly packing of the PG molecules at an interface does not seem to occur. It is possible that sufficient PG could enter the lipid layers of mitochondrial membrane and thus disrupt the close aligment of the natural components.

McNamara and his colleagues (1980) have compared the order of potency for mechanical contraction in bovine intrapulmonary vein with the order of potency for releasing calcium from the mitochondria fraction of the same tissue. 11,9-Epoxymethano PGH2 is at least 1,000 times more active than PGA2 and other PGs in producing an increase in tension while PGA2 is the most potent calcium-releasing agent in mitochondria and 11,9-epoxymethano PGH2 released only about half as much calcium as PGA2. PGA2 can be formed by spontaneous dehydration of PGE2. In PGE2-sensitive preparations PGA2 has been found to be very weakly active relative to PGE2. It is generally considered that it is unlikely that PGAs occur naturally in mammals. These findings seem to support the idea that PG release of calcium from mitochondria is not a receptor-mediated process. Since

it is believed that mitochondria originate from a different source than the rest of the cell, and have their distinct genetic system, we can not exclude the possibility that PGAs might be synthesized in the mitochondria and play a certain role in biological activity. PGA-series have been found in the Florida sea whip, Plexaura homomalla, and in varieties of P. homomalla collected from various locations in the Caribbean area in surprisingly large amounts.

In fact, it has been found that prostaglandin synthetase activity is present in mitochondrial membrane subfractions from rabbit kidney medulla (Erman, Azuri & Raz (1983). Both outer and inner membrane fractions of the medullary mitochondria catalyzed PGE2 biosynthesis from arachidonic acid. The specific activity of prostaglandin synthetase in the outer membrane is 9-10 fold higher than in the inner membrane. Rao and Mitra (1982) have reported that a variety of intracellular organelles, as well as outer cell-membranes of bovine corpora lutea, contain PGE1 and PGF2a binding sites; mitochondria free from lysosomes do not exhibit intrinsic PGE1 or PGF2a binding. However, the other PGs were not tested. Thus, up to now, it is uncertain whether PG receptors exist in mitochondria and what role PGs may play in the organelle. However, one can assume that PGs might act on membrane receptors and release membrane-bound calcium as "trigger calcium" or PGs may operate a special type of calcium channel resistant to calcium channel blockers, and at the same time facilitate the release of calcium from intracellular calcium stores, since McNamara and his colleagues (1980) showed that the threshold concentration

for calcium release by PGA2 and 11,9-epoxymethano PGH2 was quite low, between 0.04-0.2 and 0.07-0.3 μM PG/mg protein, respectively. Calcium antagonists may aid the facilitation. Since the effect of noradrenaline, which also can mobilize intracellular calcium, was not potentiated by calcium antagonists, it is reasonable to assume that the "trigger calcium" may pass through a special route to reach mitochondria, and noradrenaline utilizes intracellular calcium from different sources than PGs.

Most agonists which are thought to act through calcium are also capable of stimulating the formation of cyclic GMP. It has been suggested that cyclic GMP may act as a second messenger. Thus, there is a possibility that PGs may stimulate sythesis of cyclic GMP, which in turn releases calcium from mitochondria.

In order to see if inhibition of mitochondrial calcium uptake can enhance PGs action, sodium azide, which is an inhibitor of mitochondrial calcium uptake, was used. This turned out to be unsuccessful, since sodium azide, probably by inhibiting oxidative phosphorylation, depresses the contraction induced by 11,9-epoxymethano PGH2. This agrees with the findings that oxygen deprivation, cyanide, or azide all depressed PG action (Coceani & Wolfe, 1966; Paton & Daniel, 1967). Coceane and Wolfe (1966) also found PG action was diminished by lowering the bath temperature, with which our results accord. The enhancement of response to 11,9-epoxymethano PGH2 by calcium antagonists was attenuated by lowering the bath temperature, too. Matzushima and Gemba

(1982) showed a similar case where the PAH accumulation in the rat kidney cortical slices pretreated at 0°C for 90 min in the medium containing verapamil was unaffected. This may suggest that not extracellular (including binding to the outside cell surface) but intracellular calcium antagonists taken up from the medium give the potentiating activity. It further proves that the acting point of calcium antagonists for the potentiation is within the cell. Evidently, PGE2 analogues achieve their effect in a different way than calcium antagonists, since the enhancement by PGE2 analogues was not affected by low temperature.

The interesting findings are that RCF, YCF and hemoglobin potentiated the response to 11,9-epoxymethano PGH2. The active principles in the hemolysate and urine may be hemoglobin and urobilin. The potentiating effect does not seem to be produced by hemin or urobilin alone, since hemin at 10 μM produced no effect, and natural urobilin in the body binds to a polypeptide. It is not clear whether the effect is due to globins, the polypeptide or their combinations with pigments.

Currently, there is considerable interest in the pharmacological properties of hemoglobin (Tanishima, 1980, 1983; Wellum, Irvine & Zervas, 1980), triggered by the observation that it may be responsible for the prolonged vasoconstriction of cerebral blood vessels that occurs after cerebral hemorrhage (Echlin, 1971; Osaka, 1977; Boullin, 1980). And more recently Bowman and Gillespie (1982) have showed that both the bovine retractor penis (another

preparation which is claimed to possess NANC nerves) and the rat anococcygeus muscle contract in the presence of blood hemolysate (hemoglobin) and the hemolysate blocks the relaxation of both the bovine retractor penis and the rat anococcygeus to the inhibitory factor extracted from the bovine retractor penis, and to field stimulation of the inhibitory nerves. In the present study, verapamil and propranolol opposed the inhibitory response to field stimulation, but chlorpromazine and promethazine failed to do so. Since verapamil and propranolol at high concentrations have been proved to have local anesthetic effect (Hay & Wadsworth, 1982a; Volpi, Sha'afi, Epstein, Andrenyak & Feinstein, 1981), they may act by blocking the inhibitory nerves.

There are a number of other possible explanations for the potentiation produced by calcium antagonists. By binding to calmodulin, these drugs may activate 15-hydroxyprostaglandin dehydrogenase which is inhibited by calmodulin, and may inhibit adenylate cyclase and phosphodiesterase, in turn affecting the intracellular cAMP level. They may affect the phosphatidylinositol (PI) turnover which leads to a change in the calcium gating system, Folkert and Schlondorff (1983) have shown that trifluoperazine increases PI turnover and PG synthesis in isolated rat glomeruli. They may influence the PG transport system and increase the intracellular concentration of PGs. They may change the affinity of PG receptors. Narayanan, Lee, Newland and Khandelwal (1983) found a cytosolic protein fraction from rabbit heart cytosol which caused marked inhibition (up to 95%) of ATP-dependent calcium uptake by cardiac sarcoplasmic reticulum. And

Fournier, Crevat, Ducet and Murisasco (1983) found a fraction, isolated from urine, which had an ionophorous activity and affected mitochondrial function by releasing intramitochondrial calcium. Calcium antagonists might mimic those substances or affect their activities.

At present we can only make assumptions. Calcium antagonists have been used as inhibitory tools. Now they have been discovered to possess new properties: releasing calcium and potentiating PGs actions. It needs to be found out whether these properties are restricted to a few tissues or are a universal phenomenon. It may be worth while examining the ultramicroscopic structure and the components of membranes of the rat anococcygeus muscle, and finding out whether there is a second messenger triggering calcium release from intracellular stores. All the drugs used in this study possess multiple activities, it should be found out whether calcium channel blockers, calmodulin antagonists, and hemoglobin and urobilin produce the potentiation by an identical or different mechanism(s).

Inhibitory Actions of Calcium Antagonists

The drugs tested for this action were verapamil, D 600, nifedipine, flunarizine and trifluoperazine. All of them inhibited contraction due to both noradrenaline and high potassium.

Noradrenaline.

Results in this study show that noradrenaline manoeuvres

intracellular calcium as well as extracellular calcium, since in the absence of calcium in the medium noradrenaline still caused contraction though a higher concentration of noradrenaline was required, and verapamil at 20 μM had no effect on the contraction. This is in agreement with work by Hudgins and Weiss (1968), Hudgins (1969), van Breemen (1969), and Deth and van Breemen (1974). They have demonstrated that isolated rabbit aortic strips retain their ability to contract phasically upon exposure to noradrenaline, even after being washed in calcium-free buffers to remove any extracellular calcium. It was proposed that this noradrenaline-induced contraction is mediated by the release of intracellular calcium into the smooth muscle myoplasm (Deth & van Breemen, 1974, 1977).

It is a general belief that contraction elicited by noradrenaline is mediated by alpha-adrenoceptors. Alpha-adrenoceptors can be divided into the alpha1- and alpha2-subtypes. Presynaptic alpha-adrenoceptors are almost exclusively of the alpha2-subtype. But, both alpha1- and alpha2-adrenoceptors can occur in comparable numbers at postsynaptic sites. Stimulation of postsynaptic alpha1- and alpha2-adrenoceptors by their selective agonists causes contraction in smooth muscle. Recently, van Zwieten, van Meel and Timmermans (1983) have shown that vasoconstriction induced by stimulation of vascular postsynaptic alpha2-adrenoceptors, using selective agonists, is reduced by calcium channel blockers through a noncompetitive mechanism, while vasoconstriction evoked by selective excitation of vascular alpha1-adrenoceptors remains

virtually uninfluenced by calcium channel blockers. Similar results were shown by Muller-Schweinitzer (1983) using canine saphenous veins where nifedipine inhibited the venoconstrictor response to the alpha-2-agonist guanfacine, leaving that to the alpha-1-agonist phenylephrine unchanged. Since Fleckenstein (1977) hypothesized that the main site of action of these drugs is on the plasma membrane, these findings seem to imply that alpha-2-receptor activation mobilizes extracellular calcium, while contraction induced by alpha-1-receptor agonists is due to release of calcium from intracellular stores. This prompted us to assume that rat anococcygeus muscle has both alpha-1- and alpha-2-receptors. However, the same Muller-Schweinitzer (1983) also found that in saphenous arteries and circumflex coronary arteries, nifedipine antagonized responses to both alpha-1- and alpha-2-adrenoceptor stimulation. Obviously, it is dangerous to subclassify alpha-adrenoceptors using calcium channel blockers. Fortunately, it has been claimed that on the smooth muscle cells of the anococcygeus there are two distinct alpha-adrenoceptors, alpha-1 and alpha-1_S, exist (Coates, Jahn & Weetman, 1982; Coates & Weetman, 1983). The alpha-1_S receptor is characterised by full agonist activity of Sgd 101/75 on the rat anococcygeus muscle and abolition of Sgd 101/75 action by benextramine. Also de Jonge, Mathy, Thoolen, Timmermans, Wilffert and van Zwieten (1983) have reported in pithed normotensive rats that alpha-1-adrenoceptor-mediated vasoconstriction consists of two components: contraction elicited by (-)-phenylephrine and by methoxamine (alpha-1 agonists) does not need an

entry of extracellular calcium ions, while contraction induced by Sgd 101/75 seems governed by an influx of extracellular calcium. It is unknown whether this is the case with alpha1 and alpha1s receptors in rat anococcygeus muscle.

It has been demonstrated that D 600, verapamil and nicardipine, a nifedipine analogue, inhibit specific ligand (WB 4101 or prazosin) binding to alpha1-adrenergic receptors in a competitive manner with IC 50 values in the μM range (Blackmore, El-Refal & Exton, 1979; Fairhurst, Whittakar & Ehlert, 1980; Glossmann & Hornung, 1980; Atlas & Adler, 1981). However, the alpha1-adrenoceptor ligand WB 4101 also binds to calcium channels (Atlas & Adler, 1981), so inhibition of ligand binding to membrane fragments by calcium channel blockers is not necessarily equivalent to alpha-adrenoceptor blockade. Hay and Wadsworth (1983) have reported that certain alpha-adrenoceptor mediated responses in the prostatic half of the rat vas deferens were unaffected by nifedipine 14.4 μM, even though WB 4101 binding to rat brain membranes is 50% inhibited by the nifedipine analogue nicardipine at 4 μM (Atlas & Adler, 1981). Hay and Wadsworth (1980, 1981) also found methoxamine (alpha1 agonist) and BaCl2-induced rhythmic contractions in rat vas deferens are inhibited in parallel by nifedipine 2-3 μM, by verapamil 30-36 μM or by D 600 14-17 μM. They assume that the site of action of the calcium channel blockers is probably not the alpha-adrenoceptor, but the ion channel, since methoxamine acts via alpha-adrenoceptors and BaCl2 does not. In our case, nifedipine is very weak, and its pA2 value is close to

nicardipine IC50 value (4 μM) for displacing WB 4101 bound to rat brain membranes, which suggests that nifedipine may bind to alpha-adrenoceptor to produce the inhibition in rat anococcygeus muscle. However, D 600 (IC50=1.1 μM) was more potent than nicardipine in displacing WB 4101 binding to the rat brain membrane (Fairhurst, Whittaker & Ehlert, 1980; Atlas & Adler, 1981). In rat liver verapamil was very weak (IC50 = 20 μM) in inhibiting adrenaline binding (Blackmore, El-Refal & Exton, 1979). In the present study the order of potency for inhibiting noradrenaline-induced contraction is verapamil> trifluoperazine> D 600> nifedipine which is not consistent with the binding data. This indicates that these calcium antagonists may not bind to adrenoceptors, supporting the assumption made by Hay and Wadsworth (1983). It should be pointed out that the order of potency we obtained is opposite to that of Hay and Wadsworth (1981) for verapamil, D 600 and nifedipine. Therefore, it would be wise not to jump to any conclusion at present stage.

Oriowo (1982) found that the removal of calcium from the bathing fluid completely abolished the response to noradrenaline in the rat anococcygeus muscle; this is in conflict with our results. However, in his study, Tyrode's solution was used, contraction was recorded isotonically, and most importantly, higher concentrations of noradrenaline were not tried. He also showed that the contractile effect of noradrenaline was not affected by dantrolene sodium which prevents the release of calcium from the sarcoplasmic reticulum but not from other intracellular storage pools such as mitochondria (Winkle, 1976). This seems to support

our early hypothesis that mitochondria are important regulators in rat anococcygeus muscle.

There is a question: Do calcium antagonists potentiate response to noradrenaline at all? The ostensible effect of calcium antagonists could be a mixture of potentiation and inhibition. The answer seems to be "no". Nifedipine has a higher affinity for binding to alpha-adrenoceptors than have D 600 and verapamil, but verapamil showed higher potency for inhibition of noradrenaline and for potentiation of PGs, and moreover, RCF, YCF and hemoglobin which can potentiate PGs, had no effect on the response to noradrenaline.

High Potassium.

The contractile response of smooth muscle to potassium is considered to depend on calcium associated with extracellular or surface membrane pools: contractions induced by a high concentration of potassium are markedly decreased by the removal of calcium from the extracellular milieu (van Breemen, Farinas, Gerba & Mcnaughton, 1972; Deth & van Breemen. 1974; Heaslip & Rahwan, 1982). Calcium flow into the cell is believed to occur through voltage-dependent channels in the membrane. these channels can be blocked by calcium channel blockers (Bolton, 1979).

In agreement with these findings, we have found that deprivation of calcium from the extracellular milieu or adding calcium channel blockers decreases the contractile response to high potassium in rat anococcygeus muscle. Despite this, we have found that a small tonic contraction

is resistant to both calcium deprivation and calcium channel antagonists. It is not clear what causes the residual response.

There are some difficulties in studying the effect of high potassium since potassium can cause release of neurotransmitters from nerve endings. The rat anococcygeus muscle has a dense adrenergic innervation and it is possible that the response induced by high potassium is caused by both potassium and noradrenaline. In order to diminish the adrenergic effect, several methods were tried. (1) 6-OH dopamine (50 mg/kg body weight) was injected intra-peritoneally into rats daily for 4 days. Then the rats were killed and preparations were set up. When challenged with tyramine, the preparations contracted markedly, which indicates that adrenergic nerve endings were still functioning. (2) Preparations were incubated with a combination of high potassium and 6-OH dopamine (400 μM) for one hour. This resulted in a severe damage to voltage-dependent calcium channels: contraction could not elicited by calcium up to 10 mM in the presence of high potassium. (3) When phentolamine was added to the organ bath, it produced a marked decrease in tension when the preparation was contracted to high potassium. Reserpine was not used since it blocks the voltage-dependent calcium channel (Casteels & Login, 1983). The method we used in this part of experiment has been mentioned in the results.

In a preparation contracted with high potassium, wash-out during the initial period decreased the tone by about 20%.

It is possible that released noradrenaline accounts for this 20% of the contraction. Since the output of noradrenaline in the bath gradually decreased, and 2 hours later the tissue contained only a small amount of noradrenaline, it is assumed that the remaining contraction is mainly due to the opening of voltage-dependent calcium channels.

In this study the order of potency for inhibiting high potassium induced contraction has been found to be verapamil> D 600> trifluoperazine> nifedipine> flunarizine. It is known that phenothiazines have alpha-adrenoceptor blocking activity. However, trifluoperazine, one of the phenothiazines, was more active than D 600 in opposing noradrenaline-induced contraction, but less potent than D 600 in inhibiting high potassium-induced contraction. Furthermore, trifluoperazine was more potent in inhibiting potassium-induced than noradrenaline-evoked contraction. This indicates that the inhibition produced by calcium channel blockers is due to blocking voltage-dependent channels, and not due to the opposing effect of noradrenaline released by high potassium.

It has been reported that in cardiovascular tissues and guinea-pig taenia coli nifedipine is about 2 orders of magnitude more potent than verapamil in blocking contractile activity produced by potassium depolarization (Andersson, Edivinsson, MacKenzie, Skarby & Young, 1983; Fleckenstein, 1977; Mikkelsen, Andersson & Lederballe Pedersen, 1979; Shimizu, Ohta & Toda, 1980; Humphrey & Robertson, 1983; Imai, 1979; Hashimoto, Takeda, Katano, Nakagawa, Tsukada,

Hashimoto, Shimamoto, Sakai, Otorrii & Imai, 1979; Spedding, 1982). However, our results show that verapamil is more effective than nifedipine in blocking high potassium-induced contraction in rat anococcygeus muscle. Nachsen and Blaustein (1979) showed similar results in rat brain synaptosomes where verapamil and D 600 were more potent than nifedipine in inhibiting calcium uptake evoked by potassium depolarization. Hay and Wadsworth (1982b) have suggested that receptor-operated calcium channels exist in two sub-types, one of which is blocked by nifedipine, and both are blocked by verapamil, D 600 and flunarizine. It is well known that calcium channel blockers such as verapamil and D 600 possess different pharmacological characteristics from the dihydropyridine family. For example, verapamil non-specifically blocks the fast calcium channel as well as the slow calcium channel, while nifedipine analogues just act on the latter (Quirion & Pert, 1982). It is unknown whether the nifedipine-resistant channel is the fast calcium channel, but our results indicate that verapamil and D 600 act on a different channel in rat anococcygeus muscle from the nifedipine-sensitive channel in guinea pig taenia coli. Interestingly, with rat anococcygeus muscle in the presence of guanethidine nerve stimulation produces mechanical inhibition but little or no hyperpolarization, while large hyperpolarizations in the guinea-pig taenia from stimulating the NANC nerves are seen. It is worth while finding out whether these findings are related to the differences between calcium channels in these two tissues.

It is noteworthy that D 600 displayed a biphasic pattern in

displacing a radiolabelled dihydropyridine compound in guinea pig brain membranes (Glossman, Ferry, Lubbecke, Mewes & Hofmann, 1982; Gould, Murphy, Reynolds & Snyder, 1983). This may indicate the nifedipine-sensitive calcium channels are not homogenous or that high concentrations of D 600 cause an allosteric effect to facilitate dihydropyridine binding.

Massingham (1973) and Nguyen Duong and Brecht (1977) demonstrated in isolated vessels that verapamil, D 600 and nifedipine relaxed high potassium-induced contractions more effectively than contractions evoked by noradrenaline. On the other hand, Mikkelsen, Addersson and Lederballe Pedersen (1979) reported that in isolated human mesenteric arteries and veins, verapamil is equally potent in relieving contractions induced by high potassium and noradrenaline, a finding that accords with our findings. This appears to indicate that noradrenaline uses the same type of calcium channel as potassium in the rat anococcygeus muscle. However, the preparations where the voltage-dependent calcium channel was damaged by combination of high potassium and 6-OH dopamine, were still sensitive to low concentration (1.0 μM) of noradrenaline, and contractions induced by noradrenaline were blocked by phentolamine. It may suggests that noradrenaline has its own calcium channels.

In this study, trifluoperazine inhibited the high potassium-induced contraction. It has been found that pimozide and trifluoperazine inhibited potassium-induced contraction (Spedding, 1982; Gould, Murphy, Reynolds &

Snyder, 1983). Elias and Boyer (1979) demonstrated that chlorpromazine (10-50 μM) produced solid gel formation with filamentous actin and that a high concentration of chlorpromazine (500 μM) inhibited polymerization of actin. However, the pA2 value for trifluoperazine to inhibit potassium induced contraction in our study is equivalent to about 1.0 μM, lower than the concentrations Elias and Boyer used. Furthermore, Karaki, Murakami, Nakagawa, Ozaki and Urakawa (1982) have shown that chlorpromazine may be a voltage-dependent calcium channel blocker in rabbit aorta (IC 50=2.3 μM) in which the adventitial layer has been removed to avoid the effects of endogenous catecholamines, and in guinea pig taenia coli (IC 50=2.9 μM). Some other alpha-blockers, rauwolscine and corynanthine were shown to block voltage-dependent calcium channels and depress calcium influx (Godfraind, Miller & Socrates Lima, 1983). It can be concluded that trifluoperazine inhibits potassium-induced contraction by acting on voltage-dependent calcium channels in the rat anococcygeus muscle.

In this study, it was found that propranolol and phentolamine inhibited potassium-induced contraction. It is possible that they produced the effect by inhibiting voltage-dependent calcium channels. It has been proposed that propranolol is a calcium channel blocker (Rokutanda, Araki, Sakanashi, 1983).

The depressing effect of the high concentration (100 μM) of verapamil on the response to 11,9-epoxymethano PGH2 might be due to its damaging effect on the contractile mechanism as

occurs with a high concentration of chlorpromazine.

As we can see, when high concentrations of calmodulin antagonists and calcium channel blockers are used, the distinction between these two groups of drugs is obscure: one group can act on the other group's site. Therefore, we have been using 'calcium antagonists' to describe these drugs.

Prostanoids and Adrenergic Stimulation

It has been postulated that PGE types play an important role in modulating the activity of the adrenergic nervous system and vascular reactivity to noradrenaline (Hedqvist, 1977; Neri Serneri, Masotti, Poggesi & Galanti, 1980; Stjarne, 1978). PGE series reduce the vasoconstrictor response to sympathetic nerve stimulation in subthreshold concentrations (Armstrong, Thirsk & Salmon, 1979; Malik, 1978). On the other hand, Greenberg, Kadowitz, Diecke and Long (1973) observed that superfused mesenteric arteries and veins showed greater contractile responses to noradrenaline in the presence of PGE1 and PGE2, and Malik, Ryan and McGiff (1976) found that PGE2 potentiated noradrenaline vasoconstriction in rat and rabbit mesenteric arteries.

This study shows that in rat anococcygeus muscle 11,9-epoxymethano PGH2 facilitates, but PGE2 reduces, the release of noradrenaline, a finding which is consistent with previous work by Al Timini, Bedwani & Stanton (1978); and both 11,9-epoxymethano PGH2 and PGE2 potentiate response to noradrenaline. The final effect of PGE2 on field stimulation

depends on the concentration of PGE2 and stimulation frequency.

The potentiating effect of calcium antagonists on response to PGs is not due to blocking the inhibitory nerve since chlorpromazine and promethazine enhanced 11,9-epoxymethano PGH2, but produced no effect on the inhibitory response when preparations were contracted by 11.9-epoxymethano PGH2. Finally, the inhibitory response seems to be calcium- and temperature-dependent.

General Discussion

Reference citations : PP355-370

GENERAL DISCUSSION

Receptor differentiation for PG receptors is a difficult task, since (1) many pharmacological preparations contain several different receptors, often giving rise to opposing actions and one PG can produce both excitatory and inhibitory responses in the same preparation; (2) subthreshold concentrations of one PG can potentiate or inhibit other PG effects; (3) PGs trigger or inhibit release of other autacoids or neurotransmitters; (4) PGs potentiate or oppose the actions of other neurotransmitters or autacoids, particularly when PGs themselves do not produce visible effects; (5) PGs stimulate synthesis of PGs; (6) PGs can be metabolized by tissues; (7) certain highly unstable PGs decay spontaneously in neutral aqueous solution.

In this study we have made some efforts to characterize PG receptors using certain preparations and a limited number of available drugs.

Characteristics of PG Receptors

PGE2 Receptors.

As has been shown, PGE2 receptors can be divided into two subtypes: subtype I mediates contraction in the bovine iris sphincter muscle, the rat gastric fundus and anococcygeus muscle and the guinea-pig trachea (Jones, R.L., Personal Communication); subtype II mediates relaxation in the cat

trachea muscle, and vasodepression in the dog hind limb in vivo. Subtype I receptor is characterized by (1) high activity of ICI 80205 and 16,16-dimethyl PGE2, (2) partial agonist activity of ZK 36374, (3) inability of EP 045 and EP 116 to block the response to PGE2 analogues , and (4) low activity of ICI 81008, PGD2, 11,9-epoxymethano PGH2 and PGI2. Subtype II receptor is distinguished by the high potency of PGE2 but the low activity of 16,16-dimethyl PGE2 and ICI 80205, and the inactivity of both ZK 36374 and PGI2.

At the present stage we cannot say that every subtype I receptor mediates excitatory effects while every subtype II receptor mediates inhibitory effects. Indeed, the definition of receptors on the basis of the type of biological response produced is totally unacceptable to many pharmacologists. In fact, data from Hedqvist (1976) showed that in the superfused guinea-pig vas deferens 16,16-dimethyl PGE2 was about 20 times more potent than PGE2 in inhibiting release of (3H)-noradrenaline induced by transmural stimulation. However, this effect might not be due to activation of adenylate cyclase. It has been suggested (Gutman & Boonyaviroj. 1979) that in adrenal medulla PGE2 inhibits catecholamine secretion through inhibition of adenylate cyclase; this causes a fall of cellular cyclic AMP resulting in reduced release of calcium from intracellular stores and reduced free cytoplasmic calcium. Another case is: 16,16-dimethyl PGE2 is 40-100 times more potent than PGE2 in inhibiting gastric secretion of acid and pepsin and in preventing the formation of peptic ulcers in laboratory animals (Main & Whittle, 1975). However, these are in vivo

experiments and the much slower oxidation of 16,16-dimethyl PGE2 by 15-hydroxy- prostaglandin dehydrogenase (15-hydroxy-PGDH) is thought to contribute to its high activity. Furthermore, it has been demonstrated that exogenous PGE2 analogues produces cytoprotection in the gastric mucosa by stimulating gastric bicarbonate secretion (Johansson, Aly, Nilsson & Flemstrom, 1983). And PGE2 also has a potent central antisecretory action in controlling gastric secretion (Puurumen, 1983). Apparently, when differentiating receptors, one should make sure a single response is observed, and metabolic and penetration factors are precluded.

PGF2α Receptor.

ICI 81008 seems to be a selective and highly active PGF2a mimetic. The high activity of ICI 81008, together with the low activity of 16,16-dimethyl PGE2 and the weak antagonism by EP 116, EP 092 or EP 045 can indicate the existence of the PGF2a receptor in a preparation. The partial agonist activity of 15-oxo EP 011 may give further support. PGI2 and ZK 36374 are very weak agonists on PGF2α receptor sites. PGD2 can mimic PGF2α contractile action in reasonable concentrations. We have not found a suitable isolated preparation with the PGD2 receptors to test the selectivity of ICI 81008 on the PGD2 receptor site. However, Jones (1978) has shown that ICI 81008 is very weak agonist on a PGD2 receptor system mediating arteriolar constriction in the sheep in vivo.

One has to keep in mind that the label of selectivity on a

compound may lead to a pitfall ---- that of the circular reasoning. Selective agonists are classified according to tissue response and tissue receptors mediating these responses are classified with selective agonists. The potential for a damaging circularity in this matter is evident. A response to an agonist classified as being selective for a receptor does not necessarily indicate that the receptor type exists in the preparation, since this requires the assumption of absolute selectivity for the agonist, if other receptors are to be excluded from consideration. Often selective labels can be misleading, but corroborating evidence from studies with selective and competitive antagonists can help clarify the issue and break the vicious circle. Therefore, it is necessary to re-examine the selectivity of ICI 81008 on different preparations, and not to draw the final conclusion before a selective PGF2a antagonist is available.

Analysis with selective antagonists can be of value in confirming receptor analyses by agonists and, in many cases, the data may be relatively simple to interpret. However, mixtures of receptors may lead to complications. For example, suppose a tissue contains two types of receptor and an agonist which can act on the two types of receptor is used. The estimated Kd value of an antagonist of one of the receptor types will not conform to the real value. The difference is ascribed to the activation of the antagonist-resistant receptors by the agonist. It can be seen from this that the estimate of the potency of the antagonist depends on the selectivity of the agonist, the relative importance of the 2 receptor types and the

selectivity of the antagonist. Thus, there is a danger of a double circularity in that the results depend not only upon the selectivity of the agonist, but also upon that of the antagonist.

Therefore, one has to remember that selectivity is relative and a receptor agonist or antagonist can act on other receptors, or even behave as a channel blocker at high concentrations. It will be wise to use different approaches to define receptors in a preparation, to re-examine the selectivity in different preparations and not to stick to the rigid selective labels.

A given agonist may have a high affinity but low efficacy for a receptor. For example, impromidine, a selective histamine H2-receptor partial agonist, is more potent, but less efficacious than histamine (Parsons & Sykes, 1980). Other examples of low efficacy with high affinity can be found in a series of alpha-adrenoceptor agonists related to clonidine (Ruffolo, Waddell & Yaden, 1980) and dobutamine ----- a drug with 25 times of the affinity but one-fortieth of the efficacy of noradrenaline for alpha-adrenoceptors (Kenakin, 1981). These facts prompted us to suspect that a different subtype of the PGF2a receptor might exist in the rat gastric fundus from that in the dog, cat and horse iris sphincter muscle, and ICI 81008 could behave as a potent partial agonist on the former preparation. Up to now, there is no evidence supporting this assumption, and the lower maximum response of ICI 81008 seems to be due to its weaker PGE2-like activity compared with PGF2a in a system

containing both PGE2- and PGF2a-sensitive contractile systems.

PGI2 Receptor.

According to the limited data available, PGI2 receptors mediating inhibitory and excitatory effect have similar properties. ZK 36374 and PGI2 are potent compounds; PGE1 is a partial agonist; PGE2, PGF2a, PGD2 and 11,9-epoxymethano PGH2 are very weak agonists.

In human PRP, ZK 36374 was 2-5 times as potent as PGI2; in contrast, in rat PRP, PGI2 was 3-4 times as potent as ZK 36374 (Casals-Stenzel, Buse & Losert, 1983). This does not sufficiently indicate that rat platelets possesses a different type of PGI2 receptor than human platelets do, since ZK 36374 has PGE2-like activity, and this may affect its PGI2-like activity on the rat platelets.

Beside its excitatory effect on rabbit iris sphincter and guinea-pig ileum, PGI2 contracts certain blood vessels (Table D.1). Since very high doses of PGI2 had to be used to elicit the contraction, it is possible that PGI2 acted on other PG receptor sites, for example, PGE2 or PGF2a receptor sites as in the bullock, dog or cat iris sphincter.

TxA2 Receptors.

It has been demonstrated that 11,9-epoxymethano PGH2 mimics TxA2. The high activity of 11,9-epoxymethano PGH2 will indicate the existence of TxA2 receptors in a preparation. The selective TxA2 receptor antagonits EP Ø45, EP Ø92 and

Table D.1 Contractile effects of PGI_2 on certain vascular smooth muscle.

Preparation	Species	Effect (μg/ml)	Reference
Coronary artery	Man	Relaxation (< 0.3)	1
		Contraction (> 0.3)	
Coronary artery	Pig	Contraction (> 0.4)	2
Aorta	Rat	Contraction (>0.5)	3
Aorta	Rabbit	Contraction (> 1.0)	4
Umbilical artery	Man	Relaxation (< 0.3)	5
		Contraction (> 0.3)	
Pulmonary artery	Rabbit	Variable (0.01-1.0)	6
Basilar artery	Dog	Relaxation (< 0.03)	7
		Contraction (> 3.0)	
Portal vein	Rat	Contraction (> 0.1)	8
Vena cava	Rat	Contraction (> 0.5)	8

1. Ginsburg,R., Bristow,M.R., Harrison,D. & Stinson,E.B.(1980) Chest, 78,1 (Suppl.), 180-186. 2. Dusting,G., Moncada,S. & Vane,J.R.(1977) Eur.J.Pharmac. 45,301. 3.Levy,J.V.(1980) Prostaglandins 19,517-529. 4. Omini,G., Moncada,S. & Vane,J.R.(1977) Prostaglandins 14,625. 5. Pomerantz,K., Sintetos,A. & Ramwell,P.(1978) Prostaglandins 15,1035-1044. 6. Salzman,P.M., Salman,J.A. & Moncada,S.(1980) J.Pharmac. Exp.Ther. 215,240-247. 7. Chapleau,C. & White,R.(1979) Prostaglandins 17,573. 8. Levy,J.V.(1978) Prostaglandins 16,93-97.

EP 116, are a great help in confirmation.

It has been realized that TxA2 receptors exhibit considerable heterogeneity. As has been pointed out, human platelets may have a different type of TxA2 receptor than smooth muscle does; rat platelets may have a distinct TxA2 receptor from all the others. Moreover, EP 045, EP 092 and EP 116 are relatively weak in blocking responses to 11,9-epoxymethano PGH2 in the rat anococcygeus muscle; a case similar to the rabbit aorta (see Table E.12, P79). It is likely that these two preparation have a similar type of TxA2 receptor. Recently, Burke, Lefer, Nicolaou, Smith and Smith (1983) have demonstrated the species differences which exist in the responsiveness of platelets to TxA2 analogues. The stable TxA2 analogues, CTA2 and PTA2 significantly inhibited the aggregatory responses to AA or 9,11-azo PGH2 in platelets from man, dog and guinea-pig, while only PTA2 produced significant inhibition in cat platelets. The aggregatory response of PRP from rabbits was not significantly blocked by either analogue. Obviously, more work should be done in this field to differentiate subtypes of TxA2 receptor and to exclude penetration factors and the possibility of existence of PG endoperoxide receptors.

PGD2 Receptor.

Little work has been done in this study on the characterization of PGD2 receptors. The existence of PGD2 receptors is beyond question. It has been reported by Jones (1976) that in the sheep cardiovascular system, PGD2 has a distinct receptor. PGD2 is a potent vasopressor agent in the

anaesthetized sheep, being 19-145 times more active than PGF2a. Later, Jones (1978) found that the PGD2-sensitive constrictor system might also exist in the systemic arterial circulations of the pig, dog, cat, rabbit and rat. He suggests that 13,14-dihydro-15-oxo PGD2 is a potent and selective agonist on this system, and 15-oxo PGF2a is more potent than PGF2a in this system.

The PGD2 receptor system has also been found in the platelets of man, horse and sheep; activation leads to an increase in cyclic AMP and inhibition of aggregation. PGD2 receptors may also exist in the brain of various mammals influencing many neural activities, such as sleep induction (Ueno, Ishikawa, Nakayama & Hayaishi, 1982; Ueno, Honda, Inoue & Hayaishi, 1983), body temperature regulation (Ueno, Narumiya, Ogorochi, Nakayama, Ishikawa & Hayaishi, 1982), and neuroendocrine function (Kinoshita, Nakai, Katakami, Imura, Shimizu & Hayaishi, 1982), and probably in the gut (Ishizawa & Minowa, 1982).

PGA2 Binding Sites.

Tritiated PGA2 was found by Attallah, Payakkapan and Lee (1974) to have the capacity to bind to a supernatant fraction of the rabbit kidney. Studies by others confirmed these findings, but led to the suggestion that this interaction is one with a soluble enzyme which specifically degrades prostaglandins (Kuehl, Oien & Ham, 1974) rather than with a true PG receptor. As has been suggested by Kuehl, Oien and Ham (1974), the binding site is probably 15-hydroxy-PGDH, an acceptor rather than a receptor.

Recently, Asano and Ogasawara (1982) have reported that PGA1 and PGA2 are potent inhibitors of (3H)-diazepam binding to the benzodiazepine receptor (probably a chloride ionophore linked with GABA receptor) of the membranes of bovine cerebral cortex while PGB1 and PGB2 are less potent and PGD, PGE and PGF series are inactive. They suggested that PGA series could be potential candidates as endogenous ligands of the benzodiazepine receptor. However, as PGA1 and PGA2 competitively inhibited (3H)-diazepam binding with quite high Ki values, 7.1 and 15 μM, respectively, it is difficult to tell whether the binding site is a PGA receptor. Furthermore, it has been found that the protein with high affinity for PGA, present in renal cortical homogenates, binds PGA covalently through a reactive sulfhydryl group (Ham, Oien, Ulm & Kuehl, 1975) and that the high affinity binding protein in human plasma binds PGA non-covalently (Gueriguian, 1975). These indicate PGA may bind to proteins rather than PGA receptors.

PGE1 Receptor.

The existence of PGE1 receptor is questionable. However, there are several lines of evidence suggesting there may be a PGE1 receptor site distinct from PGE2 and PGI2 receptor sites.

In male Wistar rats PGI2 is about one order of magnitude more potent than PGE1 or PGE2 in systemic vasodepression effect; d,**1**-11, 15-bisdeoxy PGE1 (Figure D.1.p328)(Fried, Santhanakrishnan, Himizu, Lin, Ford, Rubin & Grigas, 1969)

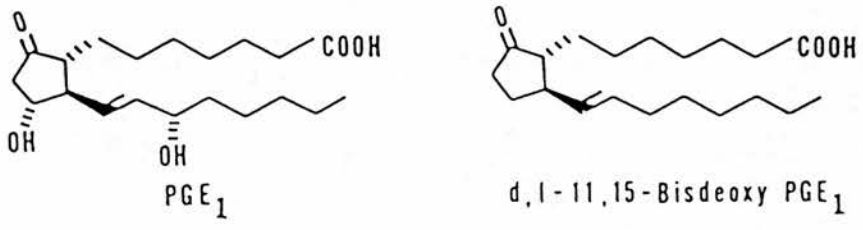


Figure D.I Chemical structures of PGE_I and d,l-11,15-bisdeoxy PGE_I.

itself had no effect on mean systemic arterial pressure. It produced reversible inhibition of PGE1-mediated vasodepression, while no significant effect was observed on PGE2 or PGI2-mediated vasodepression (Stinger, Fitzpatrick, van Dam, Ramwell & Kot, 1980). On the isolated gerbil colon preparation, d,l-11, 15-bisdeoxy PGE1 is a specific antagonist of the contractile response to PGE1, and had no agonist activity or effect on the contractile response to PGF2a or Ach (Tolman, Partridge & Barris, 1977).

PGs are known to regulate immune responses and fibrous tissue formation. It has been reported (Das, 1981) that deficiency of PGE1 and/or TxA2 and excess PGE2 seem to activate B-cells and suppress T-cell function and enhance fibrosis. Viruses can block the enzyme delta-6-desaturase necessary for PGE1 synthesis and thus depress cell-mediated immune response. Drugs which cause autoimmune disorders also seem to block PGE1 and/or TxA2 synthesis and enhance PGE2 formation, which may lead to excess auto-antibody formation. Drugs like colchicine. which can enhance TxA2 formation and the biological action of PGE1 were found to be of benefit in auto-immune disease in man and animals. But PGI2 action on the immune system has never been reported. The possibility that PGE1 produces the effect by acting on PGI2 receptor should be precluded.

As has been mentioned before, trimethoquinol reduced contraction of the rat gastric fundus to PGE1, but not PGE2 (Bennett, Jarosik, Sanger & Wilson, 1980). This indicates PGE1 may have a different acting site from PGE2.

In chick ileum, PGE1 is more potent than PGE2 for contraction and SC-19220 lacks effect on the response to PGE1 and PGE2 (Coleman, Kennedy, Levy & Penning, 1980). Since PGI2 is very active on the preparation and PGE1 is capable of acting on both PGE2 and PGI2 receptors, the possibility of a synergestic effect between PGI2 and PGE2 should be excluded. Nevertheless, a similar order of agonist potency to that in chick ileum has been described in a number of systems in which prostaglandins sensitize peripheral nociceptors (Tyers & Haywood. 1979).

The above findings suggest there is a PGE receptor system different from the PGE2 subtype I and II receptors. It merits further investigations to find out if it is another subtype of the PGE2 system or a PGE1 system.

Properties of PG Receptors

It has been suggested that prostaglandin receptors may be a magnesium- and disulfide-dependent protein (Altura & Altura, 1971, 1974; Altura, Altura & Waldemar, 1976; Greenberg & McGowan, 1981; Greenberg, Kadowitz, Long & Wilson, 1976). Similar conclusions were obtained by Johnson et al. (see Bohr, Greenberg & Bonacorrsi, 1978), who showed that the binding of PGE and PGF to uterine smooth muscle and red blood cell membranes was diminished by disulfide bond reduction with dithiothreitol and dithiobisnitrobenzoic acid. Divalent cations, e.g. calcium or magnesium ions are also essential for the binding of radiolabelled

prostaglandins to the membranes of vascular smooth muscle (Rucker & Schror, 1983).

Structure-Binding and Structure-Activity Relationships (SBR & SAR)

Soon after the first members of the prostanoid family were characterized chemically by Bergstrom (1967), and pure materials were available for biological studies, the preparation of chemically modified prostanoids or analogues began. Since then a very large number of PG analogues have been made by many research groups.

The types of analogues synthesized have expanded as the number of AA metabolites formed from prostaglandin synthetase have grown to include the endoperoxides, thromboxane, and prostacyclin. More recently the 5-lipoxygenase pathway has been further characterized to cover the hydroperoxy and hydroxy fatty acids and the leukotrienes. The reasons for preparing the analogues are as following:

(1) To provide increased metabolic stability and chemical stability. It became apparent very early that these fatty acid-based autacoids were usually quite short-lived in vivo (Samuelsson, Granstrom, Green & Hamberg 1971), although the half-life of the endoperoxides (5 min) and TxA2 (30 s) were especially noteworthy (Hamberg, Svensson, & Samuelsson, 1976). The 15-hydroxy prostaglandin dehydrogenase which is the metabolic enzyme which degrades the prostaglandins to the relatively inactive 15-keto derivatives is widespread and largely responsible for the short-lived effects (Anggard

& Samuelsson, 1966). It is clear that one needs longer-lasting compounds which can escape the rapid inactivation in vivo and in vitro for basic experimental study and clinical use.

(2) To find agents with greater selectivity.

(3) To provide selective receptor antagonists.

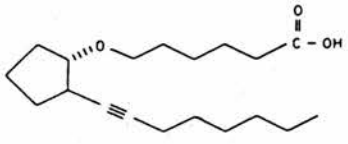
(4) To synthesize enzyme inhibitors.

PG Receptor Antagonists.

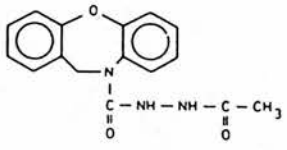
The use of receptor antagonism to establish the identity of receptors has developed only slowly in the prostanoid field since the main organic synthetic effort has been devoted to the development of long-acting, stable PGE, PGF, and PGI analogues. Studies of the actions of natural substances such as Ach, histamine, and catecholamines have been greatly aided by the availability of specific, competitive pharmacological antagonists of these substances, and isolated smooth muscle preparations are powerful tools for studying such antagonists. From a pharmacological standpoint. the optimal antagonist should be specific, potent, reversible, and possess no agonist activity and have no activity against PG synthetase.

In the early stage, three main types of substances (Figure D.2) were used as pharmacological antagonists of the prostaglandins. The anti-prostaglandin activities of each of these classes of compounds was first demonstrated on isolated preparations of gastrointestinal smooth muscle.

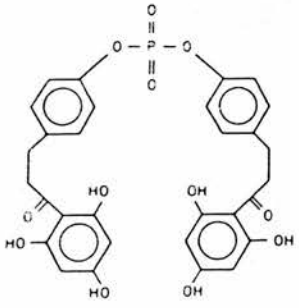
In 1969, Fried, Santhanakrishnan, Himizu, Lin, Ford, Rubin



7-Oxa-prost-13-ynoic acid



SC-19220



Di-4-phloretin phosphate

Figure D.2 Chemical structures of 7-oxa-prost-13-ynoic acid, di-4-phloretin phosphate and SC-19220

and Gringas reported that they had sythesized a series of 7-oxaprostaglandin analogues. Among them compounds with 15-hydroxy substituents were reported to be mixed agonists and antagonists (Fried, Lin, Mehra, Kao & Dalven, 1971), and the 15-nor compound with a 13,14-triple bond (7-oxa-13-prostynoic acid) has become known as the prototype prostaglandin antagonist of this series. Flack (1970) found that 7-oxa-13-prostynoic acid was the only 7-oxaprostaglandin analogue out of 15 tested that would specifically inhibit prostaglandin-induced contractions on isolated gerbil colon. It did not, however, specifically inhibit prostaglandin-induced contractions on isolated guinea-pig ileum (Flack, 1970; Bennett & Posner, 1971), rabbit jejunum (Flack, 1970), or on human stomach, ileum, or colon (Bennett & Posner, 1971). On isolated rat stomach strips 7-oxa-13-prostynoic acid produced contraction, indicating prostaglandin-like agonist activity (Bennett & Posner, 1971). Prostaglandin antagonism with 7-oxa-13-prostynoic acid has been demonstrated best in experiments showing inhibition of prostaglandin-stimulated formation of cyclic AMP, and it has been found to bind weakly to prostaglandin binding sites that are proposed as prostaglandin receptors (Bennett, 1974; Sanner, 1974; Sanner & Eakins, 1976). Baudouin-Legros, Meyer and Worcel (1975) concluded that the effect of 7-oxa-13-prostynoic acid was different on proestrous rat uteri than on the metestrous uteri. They found that on the proestrous uterus the compound inhibited PGF2a-induced stimulation without reducing the maximum response, but on the metestrous uterus it reduced

the maximum response to PGF2a. This may suggest competitive antagonism on the proestrous uterus and noncompetitive inhibition on the metestrous uterus. 7-Oxa-13-prostynoic acid also inhibited spontaneous contractions and those produced by angiotensin II on the proestrous uterus, thus indicating either a non-specific inhibitory effect of the compound or that prostaglandins are involved in these contractions.

Two non-prostanoid compounds, the polymeric compound polyphloretin phosphate (PPP) and SC-19220, have also been noted to attenuate responses to PGs. PPP is a mixture of polyester polymers that affects the activity of many enzymes, including hyaluronidase, alkaline phosphatase, and urease (Eakins & Sanner, 1972). Intra-arterial administration of PPP blocks changes in ocular membrane permeability (Beitch & Eakins, 1969), that have been associated with a mixture of PGs (Eakins & Sanner, 1972).

Unlike SC-19220 and 7-oxa prostaglandin, PPP 200 mg/kg does block the cardiovascular actions of PGF2a (Villanueva, Hinds, Katz & Eakins, 1972; Mathe, Strandberg & Fredholm, 1972), yet is ineffective in inhibiting the vasodepressor actions of PGE1 and PGE2 (Nakano, Prancan & Moore, 1971). Such high doses of PPP significantly decrease systemic arterial pressure (Mathe, Strandberg & Fredholm, 1972) and the compound may in fact exert its inhibiting action at sites other than the PG receptor, such as adenylate cyclase (Hynie, Cepelik, Cernohorsky, Klenerova, Skrivanova & Wenke, 1975; Kuehl & Humes, 1972).

Eakins and Karim (1970), using isolated gerbil colon preparations, found that PPP would specifically inhibit prostaglandin-induced contractions. It was thought that this compound inhibited only PGF series, but it was later found to inhibit contractions produced by PGE also (Eakins, Karim & Miller, 1970; Park & Dyer, 1973). It did not inhibit contractions produced by Ach, bradykinin, angiotensin, or 5-HT.

High concentrations of PPP were required to inhibit prostaglandin-induced contractions on human isolated gastrointestinal muscle preparations. PPP, like the other antagonists, did not inhibit the relaxant effect that prostaglandins have on circular gastrointestinal smooth muscle (Bennett & Posner. 1971).

Despite its prostaglandin inhibitory effects on most smooth muscle, PPP has stimulatory activities on some smooth muscles that are also stimulated by prostaglandins. Thus, PPP was found to stimulate rather than inhibit contractions of rat isolated stomach strips (Bennett & Posner, 1971; Adaiken & Karim, 1973). It also produced contractions or increased pendular movements of rabbit jejunum (Eakins, Karim & Miller, 1970). Low concentrations of PPP increased spontaneous contractions of human isolated jejunum, while high concentrations inhibited them (Adaiken & Karim, 1973). Since PPP inhibits metabolism of prostaglandins by 15-hydroxy-PGDH (Marrazzi & Matschinsky, 1972), this may account for its stimulatory effect on these tissues.

As mentioned above, PPP is a mixture of different weight polymers. It has been found that the prostaglandin inhibitory activity lies in the low molecular weight fractions, while the enzyme inhibitory activity is generally associated with the higher molecular weight fractions (Eakins, 1971; Bethel & Eakins, 1971). The dimer, di-4-phloretin phosphate, is considerably more active as a prostaglandin antagonist than is the mixture, polyphloretin phosphate (Eakins, Fex, Fredholm, Hogberg & Veige, 1973). Unfortunately, di-4-phloretin phosphate is also a more potent inhibitor of 15-hydroxy-PGDH (Crutchley & Piper, 1973).

It is necessary to point out in most of the preparations stated above PG receptors have not been defined and the selectivity of these two types of compounds has not been determined.

Another compound, SC-19220 (P.333), a dibenzoxazepine derivative, which was initially tested as an anticonvulsant and analgesic agent (Coyne & Cusic, 1968), specifically inhibits PGE2 induced contractions of the isolated guinea-pig ileum at low concentrations (Sanner, 1969; Bennett & Posner, 1971). Since contractions produced by bradykinin, 5-HT and Ach were not inhibited, the compound was considered to be a specific PG antagonist. Recently, Kennedy, Coleman, Humphrey, Levy and Lumley (1982) have made an intensive study on this compound. They have found that SC-19220 at 300 μM had little or no effect on responses of dog saphenous vein, rat aorta and guinea-pig lung strip to

11,9-epoxymethano PGH2 or PGE2; neither did it affect responses of dog and cat iris sphincter muscles to PGE2 or PGF2a. Therefore, SC-19220 is devoid of antagonistic actions in both TxA2 and PGF2a-sensitive preparations. On the other hand, SC-19220 caused concentration-related parallel shifts to the right of concentration-response curves for PGE1, PGE2 and PGF2a on guinea-pig ileum and guinea-pig and dog fundus where PGE2 is the most potent prostanoid. However, others have reported that in concentrations of greater than 10 μM, SC-19220 has actions apparently unrelated to blockade of prostanoid receptors (Bennett, 1974), and it has been found that higher concentrations of SC-19220 flattened the curve for PGE_2 and reduced the maximum contractions (Jones, R.L., Personal Communication). The inhibition of PG-induced contractions on rat fundus strips (Bennett & Posner, 1971) is in contrast to stimulation of this tissue produced by 7-oxa-13-prostynoic acid and by PPP. The difference is probably due to the lack of inhibitory effect of SC-19220 on 15-hydroxy-PGDH that was seen with both 7-oxa-13-prostynoic acid and PPP (Marrazzi & Matschinsky, 1972).

The above three compounds and their close relatives have presented several practical problems. For example, excessive amounts of these antagonists are required to obtain inhibition of PG action in vitro. Thus, continued efforts have been made to systematically screen new prostanoid analogues in an attempt to find more selective and effective antagonists.

PGF2a Antagonists.

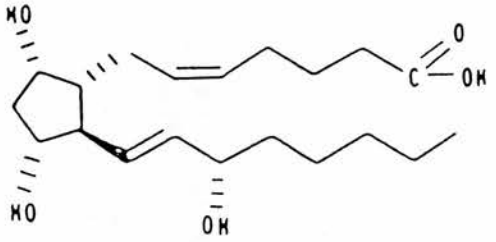
Several compounds with antagonistic activity against PGF2a have been reported.

Ceserani, Gandolfi, Longiave and Mandell (1979) suggested that 2Ø-methyl- 13,14-didehydro PGF2a might be a selective antagonist of PGF2a on rat uterus preparation; it did not show any antagonistic activity against the contractions induced by PGE2, Ach, histamine and 5-HT on guinea-pig ileum, by BaCl2 on duodenum and by PGE2, 5-HT and Ach on rat uterus. However, we have found that a similar PGF2a analogue 13,14-didehydro PGF2a is an active and full agonist on a PGF2a-sensitive preparation ---- the dog iris sphincter.

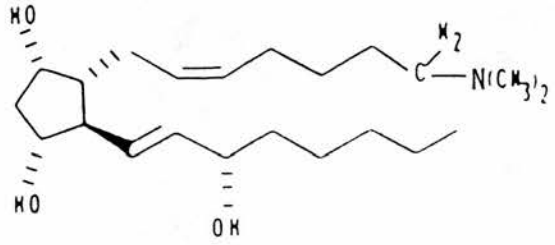
Fitzpatrick, Alter, Corey, Ramwell, Rose and Kot (1978) showed N-dimethylamine PGF2a (Figure D.3) and N-dimethylamide PGF2a antagonized the vasoconstrictor response to PGF2a in the isolated canine lung lobe, but not to AA*. Also Stinger, Fitzpatrick, Corey, Ramwell, Rose and Kot (1982) found N-dimethylamine PGF2a selectively antagonizes the pulmonary and systemic vascular responses to PGF2a in the intact rat in a dose-dependent fashion. The same strategy, that is, synthesis of the C-1 dimethylamine or dimethylamide which was used for developing the PGF2a antagonist, was also used for PGI2 but without success; this is interesting since the strategy has been successfully extended to PGE series. (Ramwell, Karanian & Foegh, 1982).

Another report from Temesvari-Major, Gruber, Tomoskozi, Kovacs and Cseh (1980) claimed that a C-8-quaternary prostanoid could be regarded as an active PGF2a antagonist

*AA - arachidonic acid



$PGF_{2\alpha}$



N-dimethylamine $PGF_{2\alpha}$

Figure D.3 Chemical structures of $PGF_{2\alpha}$ and its N-dimethylamine analogue.

in the uterus.

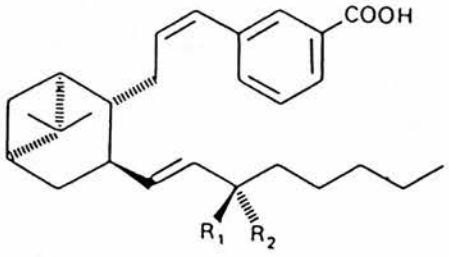
More work should be done to test the selectivity and potency of these compounds.

TxA2 Antagonists.

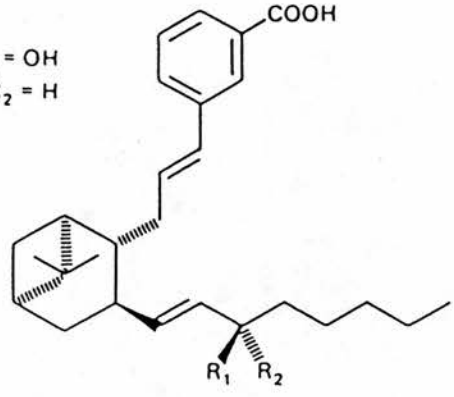
A greater number of TxA2 receptor antagonists have been synthesized. In 1978, Fitzpatrick, Bundy, Gorman and Honohan reported that 9,11-EIP (9,11-epoxyimino-prosta-5,13- dienoic acid) antagonized platelet aggregation by direct TxA2-receptor blockade. However, the compound shows agonist properties on coronary artery and rat aorta, and it also inhibits PGI2 formation in rabbit lung microsomes and BALB 3T3 cells at 10 μM. Similarly, PTA2 and CTA2 were claimed to be TxA2 receptor antagonists (Lefer, Smith. Araki, Smith, Aharony, Claremon, Magolda & Nicolaou, 1980; Nicolaou, Magolda, Smith, Aharony. Smith & Lefer, 1979), but in certain cases they showed agonist activity. Roth, Lefer, Smith and Nicolaou (1982) have synthesized six PTA2 analogues (Figure D.4) and tested their ability to antagonize CTA2-induced coronary vasoconstriction and TxA2 analogue — induced platelet aggregation. Two of the derivatives, 5C-15(S) BPTA2 and 5T-15(S) BPTA2 showed high potency in inhibiting of CTA2 induced vasoconstriction of cat coronary arteries; 5C-15(S) BPTA2 also antagonized TxA2 analogues-induced human platelet aggregation.

Harris, Phillips. Michel. Goldenberg, Heikes, Sprague and Antonaccio (1981) showed SQ 26536 (8(R), 9(S), 11(R), 12(S)-9α- homo- 9,11-epoxy- 5(Z), 13(E)- 15(S)-

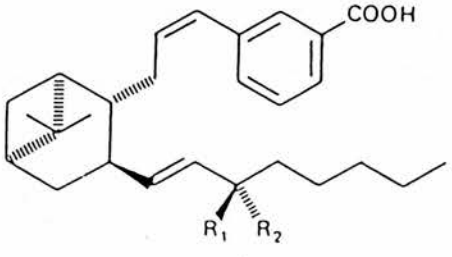
p 344



5C-15S-BPTA$_2$: R_1 = H, R_2 = OH
5C-15R-BPTA$_2$: R_1 = OH, R_2 = H



5T-15S-BPTA$_2$: R_1 = H, R_2 = OH
5T-15R-BPTA$_2$: R_1 = OH, R_2 = H



5C-15R-15Me-BPTA$_2$: R_1 = Me, R_2 = OH
5C-15S-15Me-BPTA$_2$: R_1 = OH, R_2 = Me

Figure D.4 Chemical structures of PTA$_2$ analogues.

hydroxyprostadienoic acid) is a TxA2 antagonist on human blood platelets, while SQ 26538, the 15-epimer of SQ 26536 is an agonist on the platelet TxA2 receptor (Figure D.5).

Coleman, Collington, Geisow, Hornby, Humphrey, Kennedy, Levy, Lumley, McCabe & Wallis (1981) demonstrated AH 19437 (1α (Z), 2β, 5α)- methyl 7-(2- (4- morpholinyl)- 3-oxo- 5-(phenylmethoxy) cyclopenyl)- 5-heptenoate, blocked 11,9-epoxymethano PGH2 action on guinea-pig lung strip, dog saphenous vein and rat aorta with pA2 values of 6.5, 6.0 and 5.9, respectively. The antagonistic action of AH 19437 was specific in that at 24 μM it had little or no effect on contractile responses of guinea-pig ileum to PGE2 or PGF2a, chick ileum to PGE2 or dog iris to PGF2a and at 72 μM had no effect on responses of guinea-pig lung strip to Ach or histamine or on responses of dog saphenous vein and rat aorta to KCl or 5-HT (Kennedy, Coleman, Humphrey, Levy and Lumley, 1982).

In this study we have demonstrated that EP 045, EP 092 and EP 116 are selective TxA2 receptor antagonists without any intrinsic activity. They have little effect on PGE2 and PGF2a receptor sites. These compounds will greatly facilitate study on PG receptors. The possible inhibitory activity of EP 045 on PGE2 biosynthesis is worthy of further study.

SBR & SAR for PGF2a.

It has been assumed that PGF2a assumes a "hair-pin" conformation (Rabinowitz, Ramwell & Davison, 1971). The three hydroxyl groups at C-9, 11 and 15 are all in

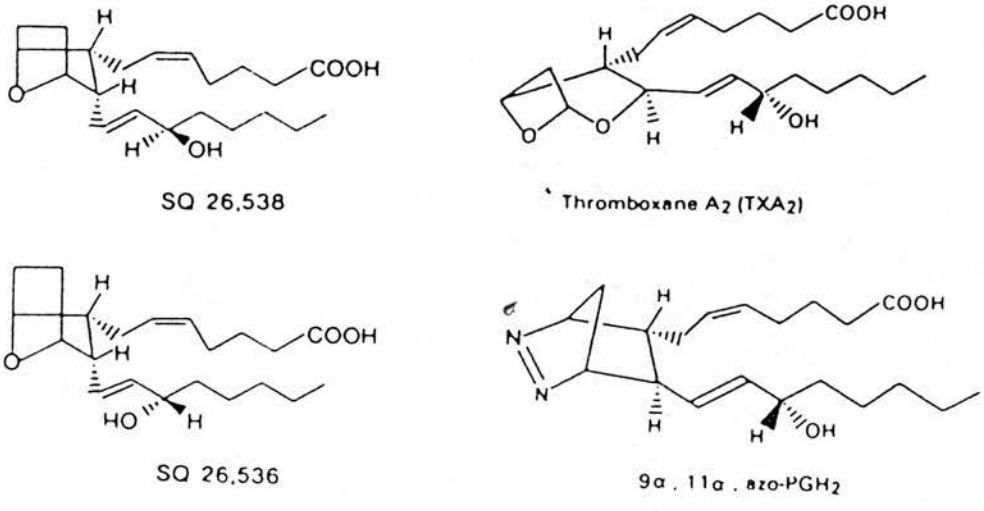


Figure D.5 Chemical structures of SQ 26538, SQ 26536, TxA2 and 9,11-azo PGH_2.

alpha-position. Any change from this hair-pin conformation or changes in chirality or inversion of any one of the hydroxyl group leads to loss of biological activity (Andersen & Ramwell, 1974; Andersen, Ramwell, Loevey & Johnson, 1976). Recently, Andersen, Imamoto, Subramanian, Picker, Ladner, De, Tyanan, Eggerman, Harker, Robertson, Oien and Rao (1981) has constrained the analogues of PGF2a to the hair-pin alignment by covalent bonding between the terminal portions of the side chains and confirmed the validity of this model in a series of new PGF syntheses. The carboxyl group and the hydroxyl group at C-15 in PGF2a are very important for the binding reaction; conversion of these groups to hydroxy (1,9,11,15- tetrahydroxyprosta-5,13-diene) and keto groups (15-keto PGF2a) increased the dissociation constants 100- and 200-fold, respectively. The hydroxyl group at C-9 and the double bond at C-5 were of intermediate importance; conversions to a keto group (PGE2) and a saturated bond (PGF1a) increased the dissociation constant 50- and 40- fold, respectively. The hydroxyl group at C-11 and the C-13,14 double bond were relatively nonimportant for the binding reaction and contractile activity; 9- and 4-fold increases in dissociation constants were observed upon oxidation to the ketone (PGD2) and reduction to a saturated bond (13,14-dihydro PGF2a), respectively (Powell, Hammarstrom & Samuelsson, 1974, 1975).

SBR & SAR for PGE2 Analogues.

The saturation of either the 5,6-trans or 13,14-cis double bonds, or removal of the 11-hydroxy group has less effect on

PGE2 subtype II than subtype I receptor. The presence of the 15(S)-hydroxy group in PGE2 is essential for high activity; substitution of a methyl group on C-15 with retention of the (S) configuration does not alter this activity; inversion of configuration. formation of a methyl ether and oxidation to a ketone at C-15 all markedly reduce its potency (Jones, 1976). On the PGE2 receptor binding site in the fetal adrenal gland PGE2 and PGE1 are very active, but the affinities of other PGs (PGF2a> PGA2> 13,14-dihydro- 15-oxo PGE2> PGD2> 6-oxo PGF1a) are 130- to 1300- fold lower (Karaplis & Powell, 1981b). TR 4752 (2-decarboxy-2-hydroxymethyl- 15-deoxy- 16 S-hydroxy- 17,17- dimethyl PGE1 seems to have high activity on PGE2 subtype II receptor but lack activity on PGE2 subtype I receptor (Gardiner & Collier, 1980). This indicates that TR 4752 may be a specific agonist on the PGE2 II receptor. 16,16-Dimethylation or 16-aryloxy substitution will increase the activity on subtype I receptor but decrease the activity on subtype II receptor. 11-Deoxy PGE1, although weaker than PGE2 on PGE2 receptor sites, is far weaker than PGE2 on PGF2a-sensitive preparations.

SAR for PGD2 analogues

Jones (1976) has proposed that compounds with the greatest selectivity on PGD2 receptor site mediating contraction must have (1) a D-type ring system, (2) 5,6-cis and 13,14-trans double bonds and (3) an oxo, (R) hydroxyl or (S) methyl ether group at C-15.

SBR & SAR for PGI2 Analogues.

The detailed relationships of SBR and SAR for the PGI2 receptor have been discussed extensively (Blair & MacDermot, 1981; Blair, Hensby & MacDermot, 1980; MacDermot, Blair & Cresp, 1981; MacIntyre, 1981). The vinyl ether moiety of PGI2 is critical, and hydrolysis results in total loss of activity (cf. 6-oxo PGF1a). The 5-6 double bond is an important determinant of activity, since alteration of the geometry at C-6 to the 5,6(E)-isomer of PGI2, saturation of the double bond to form 6β-PGI1 or a shift of the double bond to C-4-5 or C-7-8 results in markedly decreased activity. ZK 36375, a 5(Z)-stereoisomer of ZK 36374, has lower affinity than ZK 36374, the 5(E)-isomer of 6a-carba PGI2 is 30- fold more potent than the 5(Z)-isomer, and the affinity of 5,6-trans PGE2 is greater than that of the cis isomer. The higher activity of 5,6-trans PGE2 may be explained as follows. The Z-configuration of the 5,6-double bond of PGI2 and the E-configuration of the 5,6-double bond of ZK 36374 (Rucker & Schror, 1983) are important for PGI2-like activity, and more closely resembles the trans configuration of 5,6-trans PGE2 than the cis configuration of the natural isomer PGE2. One interesting anomaly is that reduction of the 5,6-trans double bond of 5,6-trans PGE2 to form PGE1 increased significantly the binding to the membrane receptor. The finding that PGE1 and PGI2 bind competitively to the same receptor suggests that the anomalous affinities (PGI2> 6b-PGI1 whereas 5,6-trans PGE2< PGE1) are not explained by the presence of multiple receptor types. It has been proposed that the alpha-side chain of PGE1 takes up a conformation that is different from PGE2 and

more closely resembles PGI2. The affinity for PGE2 is 100 times lower than that for PGE1. The 15-hydroxyl group is an absolute requirement for high affinity binding since PGE2 has a higher affinity than 15-oxo PGE2 in binding to the PGI2 receptor, and it holds true for 13,14-dihydro PGE2 and 15-oxo- 13.14-dihydro PGE2. Furthermore, the absolute configuration at C15 is critical, as the activity of the natural isomer (15(S)-OH) is lost in 15(R)-PGE2. Some structure-activity data have been obtained using analogues of PGI2 or other ring-modified derivatives in which the 6.9 α epoxy linkage is replaced by 5,9 α epoxy, 9 α,5 nitrilo, 6,9 α imino, 6,9 α epithio or 6a- carba linkages. Among them 9-deoxy- 9α,6-nitrilo PGF1 is equipotent with PGI2 as an inhibitor of platelet aggregation, while the others display reduced or zero activity. Removal of the C-13-14 double bond has little effect on activity. Addition of a trans C2-3 double bond augments potency. Transformation of 15(S) into 15(R) leads to loss of activity. The 17-methylation augments potency. 16-Phenoxy substitution of the 17.18,19,20-tetranor derivatives of PGI_2 reduces PGI2-like activity but increases other prostanoid-like activity.

MacDermot, Blair and Cresp (1981) have reported that divalent cations regulate the binding of PGI2 to its membrane receptor. Magnesium increase the binding. Proton magnetic resonance spectroscopy shows that an upfield shift of H5 and downfield shift of H15 in the presence of magnesium ions. They propose that PGI2 may exist in two or more conformational states with different Kd values in the

ligand-receptor interaction. The functionally critical substituents, the vinyl ether moiety and the 15(S)-hydroxyl group, are adjacent to protons that undergo magnesium-dependent changes in their electronic environment. These substituents allow formation of a complex between the divalent cation and prostacyclin, and the altered geometry of PGI2 satisfies the geometric constraints of the PGI2 receptor.

SAR for TxA2 Mimetics

11,9-epoxymethano PGH2 seems to be a selective TxA2 receptor agonist. But its isomer 9,11-epoxymethano PGH2 is only a partial agonist. 9,11-Azo substituent sustains the spectrum of TxA2 action. Replacing the epoxymethano group with etheno or ethano groups results in partial agonism. C-16-p-fluorophenoxy or C-16-p-chlorophenoxy substitution changes these compounds into full agonists in smooth muscle. Substitution of oxygens in the ring of TxA2 with carbon atoms keeps its full agonist activity in smooth muscle contraction but results in reduced potency in inducing platelet aggregation. The pinane structure in the ring will markedly reduce TxA2-like activity in both smooth muscle and platelet. The 15-hydroxy group is essential for platelet aggregation but not for smooth muscle contraction.

MacIntyre, Salzman and Gordon (1978) have suggested the requirements for dienoic prostaglandins to act on the TxA2/endoperoxide receptor: (1) the configuration of the molecule should be such that the side chains are out of plane with the ring, as they are in the native compounds.

The chains of PGE2, PGF2a and PGA2 are in plane and thus they cannot affect the receptor in such way as to produce aggregation. (2) Certain changes in C-11, C-15 and C-16 will achieve the required configuration and will probably favourably alter lipid solubility characteristics. The latter conclusion is supported by the effect of modified PGE2 analogues (e.g. 16,16-dimethyl PGE2)which act as TxA2. (3) the existence of two rings (bicyclic compounds) appears to be a strong and perhaps sufficient (although not sole) stimulus for affecting the site.

Methylation and Aromatic Substitution.

The discussion below is based on data from this study, Andersen, Imamoto, Subramanian, Picker, Ladner, De, Tynan, Eggerman, Harker, Robertson, Oien & Rao (1981), Jones, Wilson & Marr (1979), and Jones, Peesapati & Wilson (1982), unless otherwise stated.

When the first metabolites of the PGs resulting from oxidation of the 15-hydroxyl to a 15-ketone by the dehydrogenase had been characterized (Anggard & Samuelsson, 1966), it was decided to look at the effect of introduction of a 15-methyl group to prevent this oxidation. These compounds were synthesized and proved to have full agonist activity but with an increased biological half-life because they could not be oxidized to the corresponding 15-ketone (Bundy, Lincoln, Nelson, Pike & Schneider, 1971). Other strategies have also been used to prevent the the metabolic inactivation of the lower side chain by the 15-hydroxydehydrogenase. These have involved the concept of

steric crowding close to the 15 position by the synthesis of 16,16-dimethyl analogues such as 16,16-dimethyl PGE2 (Magerlein, DuCharme, Magee, Miller, Robert & Weeks, 1973) which are again not substrates for the enzyme and increase PGE2-like activity, but reduce PGF2a-like activity. Methylation at C-15 or C-16 position of PGF2a will increase PGF2a-like activity. The order of potency for methylated PGF2a analogues in a PGF2a-sensitive preparation is 15-Me= 16,16-diMe= PGF2a= 18,18-diMe> 17,17-diMe; the order of potency for methylated PGE2 analogues in PGF2a-sensitive preparations is PGE2 > 15-Me= 16,16-diMe> 17,17-diMe> 18,18-diMe. Also the methylation will confer TxA2-like activity on the PGE2 and PGF2a compounds.

In complete contrast to PGF2a and PGE2 receptors, 17,17-dimethylation is the best tolerated modification for retaining PGI2 potency while 16,16-dimethylation is most disruptive. 17,17-dimethylation actually improves PGI2-action specificity even for the PGE2 analogue: 17,17-dimethyl PGE2, unlike natural PGE2, is a potent anti-aggregatory agent. The disruptive influence of 16,16-dimethylation on PGI2-like action is also seen in the PGE2 analogues: 16,16-dimethyl PGE2 is an aggregating stimulus by itself (Corey, Gordon, MacIntyre & Salzman, 1977), probably through its TxA2-like activity. And 16,16-dimethylation converts PGF2a into an aggregating agent. The rat liver PGE2 receptor affinities and smooth muscle activities of the prostacyclins are also consistent with the hypothesis that 17-methylation enhances prostacyclin specific activity for both PGE and PGI

compounds while 16,16-dimethylation enhances PGE2-like activity, even for prostacyclin structures: 16,16-dimethylation results in aggregatory activity in an exo-PGI2 analogue structure has been reported (Gandolfi & Ceserani, 1981). Thus one can predict that removal of the methyl group at C-16 position of ZK 36374 and replacing it at C-17 position will decrease its PGE2-like activity and increase its PGI2-like activity.

Another tactic is to substitute the alkyl lower side chain with aromatic rings such as phenyl or phenoxy residues (Magerlein, Bundy, Lincoln & Youngdale, 1975; Crossley, 1976). Replacement of the C17-20 unit in PGD2, PGF2a, PGE2, 9,11-etheno and 9,11-ethano PGH2 with a p-fluorophenoxy or p-chlorophenoxy moiety markedly increase TxA2-like activity. Also these substitutions confer both high PGE2- and PGF2a-like activity on PGE2 or PGF2a and make PGE2 and PGF2a more versatile. The 16-m-trifluoromethylphenoxy group will increase the activity on PGF2a receptor site and give high selectivity.

Other Substituents

11-deoxyprostaglandins, although less active than the corresponding natural PGs have the advantage of relative stability and ease of synthesis. These analogues, when combined with other features in the ω-side chain (15- and 16-methyl substituents) have an interesting level of activity and selectivity (Bartmann, Beck, Lerch, Teufel, Babej, Beckel, Schoelkens & Seeger, 1979).

Other approaches in analogue synthesis has been to design compounds with substitution of the oxygen in the ring of PGH_2 and TxA_2 with nitrogen (Bundy & Baldiwin, 1978), sulphur (Nicolaou, Barnette, Gasic & Magolda, 1977; Hamanaka, Ohuchida & Hayashi, 1982), and carbon (Morton, Bundy & Nishizawa, 1979; Corey, Shibasaki, Nicolaou, Malmsten & Samuelsson, 1976; Bundy, Kimball, Robert, Aiken, Maxey, Sebek, Nelson, Sih, Miller & Hsi, 1980), to make configurationally rigid arylprostaglandins (Schaaf, Johnson, Constantine, Bindra, Hess & Elger, 1983), N-(methanesulfonyl)- 16-phenoxy prostaglandincarboxamides (Schaaf, Bindra, Eggler, Plattner, Nelson, Johnson. Constantine, Hess & Elger. 1981) and 13-aza- 14-oxo- prostaglandins (Favara, Guzzi, Ciabatti, Battaglia, Depaol, Gallico & Galliani, 1983). These compounds should be tested in preparations where PG receptors have been defined, or better in preparations with a single type of PG receptor. to estimate their potency and selectivity. Schillinger. Prior, Speckenbach and Wellershoff (1979) have reported the one of the N-methanesulfonyl-16- phenoxy compounds, sulprostone, seems to have increased affinity for PGE2 as well as PGF2a binding site.

The aim of studying receptors is to extend our knowledge of receptor theory and to discover selective and efficacious drugs. Owing to the successful synthesis of selective TxA2 receptor antagonists with high affinity the investigations of PG receptors have entered a new stage. More work remains to be done in this field in order to synthesize and screen more selective and potent agonists and antagonists for

pharmacological study and therapeutic use.

References

Part One

Adaiken,P.G. & Karim,S.M.M.(1973) J.Pharm.Pharmac. 25, 229-233

Adamson,U., Eliasson,R. & Wiklund,B.(1967) Acta.Physiol.Scand. 70, 451-452

Aharony,D., Smith.J.N., Smith.E.F., Lefer.A.M., Magolda,R.L. & Nicolaou,K.D.(1980) In: Advances in Prostaglandin & Thromboxane Research vol.6 ed. Samuelsson,B., Ramwell.P.W. & Paoletti,R. pp489-492 New York, Raven Press

Alphen,G.W.H.M. & Angel,M.A.(1975) Prostaglandins 9, 157-166

Altura,B.M. & Altura,B.T.(1971) Am.J.Physiol. 220, 938-944

Altura,B.M. & Altura,B.T.(1974) Microvasc.Res. 7, 145-155

Altura,B.M., Altura,B.T. & Waldemar,Y.(1976) Artery 2, 326-336

Ambache,N., Brummer,H.C., Rose.J.G. & Whiting,J.(1966) J.Physiol. 185, 77p-78p

Andersen,N.H. & Ramwell,P.W.(1974) Arch.Int.Med. 133, 30-50

Andersen,N.H., Eggerman,T.L., Harker,L.A., Wilson,C.H. & De,B.(1980) Prostaglandins 19, 711-735

Andersen,N.H., Imamoto,S., Subramanian,N., Picker,D.H., Ladner,D.W., De.B., Tyanan,S.S., Eggerman,T.L., Harker,L.A., Robertson,R.P., Oien,H.G. & Rao,C.V.(1981) Prostaglandins 22, 841-856

Andersen,N.H., Ramwell,P.W., Loevey,E.M.K. & Johnson,M.(1976) In: Advances in Prostaglandin and Thromboxane Research vol.1 ed. Samuelsson,B. & Paoletti,R. pp271-289 New York, Reven Press

Anggard,E. & Bergstrom.S.(1963) Acta Physiol.Scand. 58, 1

Anggard,E. & Samuelsson,B.(1966) Ark.Kemi. 25, 293

Anggard,E. & Samuelsson,B.(1964) Biochem.Pharmac. 13, 281-283

Anggard,E.(1966) Acta Physiol.Scand. 66, 509

Ansell,M.F., Caton,M.P.L., Palfreyman,M.N., Stuttle,K.A.J., Tuffin,D.P. & Walker,J.L.(1980) In: Advances in Prostaglandin and Thromboxane Research vol.6 ed. Samuelsson,B., Ramwell.P.W. & Paoletti,R. pp485-487, New York, Raven Press

Apperley,E., Feniuk,W., Humphrey,P.P.A. & Levy,G.P.(1980) Br.J.Pharmac. 68, 215-224

Apperley,E., Humphrey,P.P.A. & Levy,G.P.(1976) Br.J.Pharmac. 56, 211-221

Apperley,G.H., Coleman,R.A., Kennedy,I. & Levy,G.P.(1979) Br.J.Pharmac. 67, 412-3p

Armstrong,R.A., Jones,R.L. & Wilson,N.H.(1983) Br.J.Pharmac. 79, 953-964

Asano,T. & Ogasawara,N.(1982) Eur.J.Pharmac. 80, 271-274

Ashida,S.I. & Abiko,Y.(1979) Thromb.Haemostas. 41, 436

Attallah,A.A., Payakkapan,U. & Lee,J.B.(1974) Life Sci. 14, 1521

Attar,S., McLaughlin,J.S. & Masaitis,C.(1973) South Med.J. 66, 481-485

Avanzino,G.L., Bradley,P.B. & Wolstencroft,J.H.(1966) Br.J.Pharmac. 27, 157-163

Ball.G., Brereton,G.G., Fulwood,M., Ireland,D.M. & Yates,P.(1970) Biochem.J. 120, 709

Bartmann,W., Beck,G., Lerch,U., Teufel,H., Babej,M., Beckel,M., Schoelkens,B. & Seeger,K.(1979) In: Chemistry, Biochemistry and Pharmacological Activity of Prostanoids, ed. Roberts,S.M. & Scheinmann,F. pp194-209 Oxford, Pergamon Press

Baudouin-Legros,M., Meyer,P. & Worcel,M.(1975) Prostaglandins 9, 203-209

Beitch,B.R. & Eakins,K.E.(1969) Br.J.Pharmac. 37, 158-167

Bennett,A. & Posner,J.(1971) Br.J.Pharmac. 42, 584-594

Bennett,A. & Sanger,G.J.(1978) Br.J.Pharmac. 63, 357-358p

Bennett,A. & Sanger,G.J.(1980) J.Physiol.(Lond.) 298, 45-46p

Bennett,A. & Sanger,G.J.(1982) Br.J.Pharmac. 77, 591-596

Bennett,A. Pratt,D. & Sanger,G.J.(1980) Br.J.Pharmac. 68, 357-362

Bennett,A.(1974) In: Advances in Drug Research, vol.8 ed. Harper,N.J. & Simmons,A.B. pp83-118 London, Academic Press

Bennett,A., Eley,K.G. & Scholes,G.B.(1968) Br.J.Pharmac. 34, 630-638

Bennett,A., Hensby,C.N., Sanger,G.J. & Stanford,I.F.(1981) Br.J.Pharmac. 74, 435-444

Bennett,A., Jarosik,C., Sanger,G.J. & Wilson,D.E.(1980) Br.J.Pharmac. 71, 169-175

Bennett,A., Pratt,D. & Sanger,G.J.(1980) Br.J.Pharmac. 63, 357-358p

Bergstrom,S. & Sjovall.J.(1960) Acta Chem.Scan. 14, 1693-1700

Bergstrom,S. & von Euler.U.S.(1963) Acta Physiol.Scand. 59, 493

Bergstrom,S.(1967) In Prostaglandins, Nobel Symposium 2. ed. Bergstrom,S. & Samuelsson,B. p21 Stockholm, Almqvist & Wiksell

Bergstrom,S., Danielson,H. & Samuelsson,B.(1964) Biochim.Biophys.Acta 90, 207

Bergstrom,S., Dressler.F., Krabigh,L, Ryhage R. & Sjovall,J.(1963) Ark.Kem. 20, 63

Bergstrom,S., Eliasson,R., von Euler,U.S. & Sjovall.J.(1959) Acta Physiol.Scand. 45, 133

Bergstrom,S., Ryhage,R., Samuelsson,B. & Sjovall.J.(1963) J.Biol.Chem. 238, 3555

Bethel,R.A. & Eakins,K.E.(1971) Exp.Eye Res. 13, 83-91

Bhattacherjee,P., Kulkarni,P.S. & Eakins,K.E.(1979) Invest. Ophthalmol. 18, 172-178

Blair,I.A. & MacDermot,J.(1981) Br.J.Pharmac. 72, 435-441

Blair,I.A., Hensby,C.N. & MacDermot,J.(1980) Br.J.Pharmac. 69, 519-525

Bohr,D.F., Greenberg,S. & Bonacorrsi,A.(1978) In: Microcirculation vol.2 ed. Kaley,G. & Altura,B. pp311-348 Baltimore, University Park Press

Bonne,C., Martin,B., Watada,M. & Regnault,F.(1981) Thromb.Res. 21, 13-22

Boot,J.R., Dawson,W., Cockerill,A.F., Mallen,D.N.B. & Osborne,D.J.(1977) Prostaglandins 13, 927-932

Borisy,G.G., Olmstead.J.B., Marcum.J.M. & Alen,C.(1974) Fed.Proc. 33, 167-174

Born,G.V.R.(1962) Nature (Lond.) 184, 927-929

Born,G.V.R.(1970) J.Physiol. 209, 487-511

Botting,J.H. & Salzmann,R.(1974) Br.J.Pharmac. 50, 119-124

Botting,J.H.(1977) J.Pharm.Pharmac. 29, 708-709

Bundy.G., Lincoln,F., Nelson,N., Pike,J., Schneider,W.(1971) Annals of New York Academy of Sciences 180, 76

Bundy,G.L. & Baldiwin,J.M.(1978) Tetrahedron Letters, p1371

Bundy,G.L., Kimball,F.A., Robert,A., Aiken,J.W., Maxey,K.M., Sebek,O.K., Nelson,N.A., Sih,J.C., Miller,W.L. & Hsi,R.S.P.(1980) In: Advances in Prostaglandin and Thromboxane Research vol.6 ed. Samuelsson,B. & Paoletti,R. p355 New York, Raven Press

Bunting,S., Moncada,S. & Vane,J.R.(1976) Br.J.Pharmac. 57, 462p

Burke,S.E., Lefer,A.M., Nicolaou,K.C., Smith,G.M. & Smith,J.B.(1983) Br.J.Pharmac. 78, 287-292

Casals-Stenzel,J., Buse,M. & Losert,W.(1983) Prostaglandins Leukotrienes and Medicine 10, 197-212

Cavanaugh,A.H. & Farmworth.W.E.(1977) Life Sci. 21, 83

Ceserani,R., Gandolfi,C., Longiave,D. & Mandell,V.(1979) Prostaglandins and Medicine 2, 455-457

Chan,J.A., Nagasawa,M., Takeguchi,C. & Sih,C.J.(1975) Biochem. 14, 2987-2991

Chang,W-C. & Tai.H-H.(1983) Prostaglandins, Leukotrienes and Medicine 12, 149-157

Charo,I.F., Feinman,R.D. & Detwiler,T.C.(1976) Biochem.Biophys. Res.Comm. 72, 1462-1467

Chijimatsu,Y., Nguyen,T.V. & Said,S.I.(1977) Prostaglandins 13, 909-916

Coleman,R.A., Collington,E.W., Geisow,H.P., Hornby,E.J., Humphrey,P.P.A., Kennedy,I., Levy,G.P., Lumley,P., McCabe,P.J. & Wallis,C.J.(1981) Br.J.Pharmac. 72, 524p

Coleman,R.A., Humphrey,P.P.A., Kennedy,I., Levy,G.P. & Lumley,P.(1980) Br.J.Pharmac. 69, 265-266p

Coleman,R.A., Humphrey,P.P.A., Kennedy,I., Levy,G.P. & Lumley,P.(1981) Br.J.Pharmac. 73, 773-778

Coleman,R.A., Kennedy,I. & Levy,G.P.(1980) Br.J.Pharmac. 69, 266-267p

Coleman,R.A., Kennedy,I., Levy,G.P. & Penning,C.(1980) Br.J.Pharmac. 70. 89p

Comaty,J.E., Ehrenpreis,S. & Greenberg,J.(1976) Pharmacologist 18. 225

Cooper.B. & Ahern,D.(1979) J.Clin.Invest. 64, 586

Cooper.B., Schafer,A.I., Puchalsky,D. & Handin,R.I.(1979) Prostaglandins 17, 561

Corey,E.J., Gordon,J.L., MacIntyre,D.E. & Salzman,E.W.(1977) Br.J.Pharmac. 59 446-7 p

Corey,E.J., Shibasaki,M., Nicolaou,K.C., Malmsten,C.L. & Samuelsson,B.(1976) Tetrahedron Letters, p737

Coyne,W.E. & Cusic,J.W.(1968) J.Med.Chem. 11, 1158

Cranefield,P.F., Aronson,R.S., Wit,A.L.(1974) Circulation Res. 34, 204-213

Crawford,C.G., van Alphen,G.E.H.M., Cook,H.W. & Lands,W.E.M.(1978) Life Sci. 23, 1255-1262

Crossley,N.S.(1976) Prostaglandins. Chemistry and Industry p334

Crutchley,D.J. & Piper,P.J.(1973) Naunyn-Schmiedebergs Arch.Pharmac. 279 (Suppl.R),20

Das,U.N.(1981) Int.J.Tiss.Reac. III(2), 89-94

Dazod,A., Morera,H.M., Bertrand,J. & Saez,J.M.(1974) Endocrinology 95, 352

Deth,R. & van Breemen,C.(1974) Pflugers Arch. 348, 13-22

Deth,R. & van Breemen,C.(1977) J.Membr.Biol. 30, 363-380

Donatsch,P., Lowe,D.A., Richardson,B.P. & Taylor,P.(1980) Br.J.Pharmac. 70, 355-362

Dukes,M., Russell.W. & Walpole,A.L.(1974) Nature (Lond.) 250, 330-331

Eagling,E.M., Lovell,H.G. & Pickles,V.R.(1976) Br.J.Pharmac. 44, 510-516

Eakins,K.E. & Karim.S.M.M.(1970) Life Sci. 9, 1-5

Eakins,K.E. & Sanner,J.H.(1972) In: The Prostaglandins, Progress in Research, ed. Karim,S.M.M. p261 London, Medical and Technical Pub.Co.Ltd.

Eakins,K.E.(1971) Ann.N.Y.Acad.Sci. 180, 386-395

Eakins,K.E., Fex,H., Fredholm,B., Hogberg,B. & Veige,S.(1973) Adv.Biosci. 9, 135-138

Eakins,K.E., Karim.S.M.M. & Miller,J.D.(1970) Br.J.Pharmac. 39, 556-563

Eliasson,R.(1957) Acta Physiol.Scand. 39, 141

Eliasson,R.(1959) Acta Physiol.Scand. 46 (Suppl.158),1

Eliasson,R., Brzdekiewicz,Z. & Wiklund,B.(1969) In: Prostaglandins, Peptides and Amines ed. Mantegazza,P. & Horton,E.W. pp57-64 London, Academic Press

Favara,D., Guzzi,U., Ciabatti,R., Battaglia,F., Depaoli,A., Gallico,L. & Galliani,G.(1983) Prostaglandins 25, 311-321

Feinman,R.D. & Detwiler,T.C.(1974) Nature (Lond.) 249, 172-173

Fitzpatrick,F.A., Bundy,G.L., Gorman,R.R. & Honohan,T.(1978) Nature (Lond.) 275, 764-766

Fitzpatrick,T.M., Alter,I., Corey,E.J., Ramwell,P.W., Rose,J.C. & Kot,P.A.(1978) J.Pharmac.Exp.Ther. 206, 139-142

Flack,J.D.(1970) Recent Prog.Horm.Res. 26, 174-179

Flower,R.J. & Blackwell,G.J.(1976) Biochem.Pharmac. 25, 285

Fried,J., Santhanakrishnan,T.S., Himizu,J., Lin,C.H., Ford,S.H., Rubin,B. & Grigas,E.O.(1969) Nature (Lond.) 223, 208-210

Fried.J., Lin,C.H., Mehra,M.M., Kao,W.L. & Dalven,P.(1971) Ann.N.Y.Acad.Sci. 180, 38-63

Gaion,R.M. & Tremto,M.(1983) Br.J.Pharmac. 80, 279-286

Ganatra,V.M., Dhumal,V.R., Bhatt,J.D. & Sachdev,K.S.(1979) Arch.Int.Pharmacodyn. 240, 203-213

Gandolfi,C.A. & Ceserani,R.(1981) In: Prostaglandins and Cardiovascular Disease, Atheroscler.Rev. 8, 183

Gardiner,P.J. & Collier,H.O.J.(1980) Prostaglandins 19, 819-841

Gerrard,J.M., Peterson,D.A., Townsend,D. & White,J.G.(1976) Circ. 196 (Suppl. II),53-54

Gerrard,J.M., Townsend,D., Stoddart,S., Witkop,C.J. & White,J.G.(1977) Am.J.Pathol. 86, 99-115

Gerrard,J.M., White.J.G. & Peterson,D.A.(1978) Thromb.Haemos. 40, 224-231

Goldblatt,M.W.(1933) J.Soc.Chem.Ind.(Lond.) 52, 1056-1057

Gorman,R.R. & Miller,O.U.(1979) Biochim.Biophys.Acta 323, 560

Gorman,R.R., Sun,F.F., Miller,O.V. & Johnson,R.A.(1977) Prostaglandins 13, 1043-1054

Greenberg,S. & McGowan,C.(1981) In: Proceedings of the Symposium on the Prostaglandins in the Microcirculation ed. Kaley,G. Baltimore, University Park Press

Greenberg,S., Kadowitz,P.J., Long,J.P. & Wilson,W.R.(1976) Circ.Res. 39, 66-76

Gryglewski,R.J., Korbut.R. & Ocetkiewicz,A.(1978) Nature 273, 765

Gryglewski,R.J., Bunting,S., Moncada,S., Flower,R.J. & Vane,J.R.(1976) Prostaglandins 12, 685-714

Gueriguian,J.A.(1975) Fed.Proc. 30, 791

Gutman,Y., Boonyaviroj,P.(1979) Eur.J.Pharmac. 55, 129-136

Hamanaka,N., Ohuchida,S. & Hayashi,M.(1982) In: V International Conference on Prostaglandins, Florence, Italy. 132p

Hamberg,M. & Fredholm,B.B.(1976) Biochim.Biophys.Acta 431, 189-193

Hamberg,M. & Samuelsson,B.(1973) Proc.Natl.Acad.Sci.U.S.A. 70, 899

Hamberg,M., Hedqvist,P., Strandberg,K., Svesson,J. & Samuelsson,B.(1975) Life Sci. 16, 451-462

Hamberg,M., Svensson,J. & Samuelsson,B.(1974) Proc.Natl.Acad.Sci.U.S.A. 71, 3824

Hamberg,M., Svensson,J. & Samuelsson,B.(1975) Proc.Natl.Acad.Sci.U.S.A. 72. 2994

Hamberg,M., Svensson,J., Wakabayashi,T. & Samuelsson,B.(1974) Proc.Natl.Acad.Sci.U.S.A. 71, 345

Hamberg,M., Svesson,J. & Samuelsson,B.(1976) In: Advances in Prostaglandin and Thromboxane Research Vol.1 ed. Samuelsson,B. & Paoletti,R. pp19-27, New York, Raven Press

Hamberg.M., Hedqvist,P., Strandberg,K., Svesson,J. & Samuelsson,B.(1975) Life Sci. 16, 451-462

Hammarstrom,S.(1983) Ann.Rev.Biochem. 52, 355-377

Ham,E.A., Oien,H.G., Ulm,E.H. & Kuehl,F.A.Jr.(1975) Prostaglandins 10, 217-229

Harris,D.N., Phillips,M.B., Michel,I.M., Goldenberg,H.J., Heikes,J.E., Sprague,P.W. & Antonaccio,M.J.(1981) Prostaglandins 22, 295-307

Hedqvist,P.(1976) In: Advances in Prostaglandin and Thromboxane Research vol.1 ed. Samuelsson,B. & Paoletti,R. pp357-363 New York, Raven Press

Holmsen,H. & Weiss,H.J.(1979) Ann.Rev.Med. 30, 119

Horton,E.W. & Jones,R.L.(1974) Br.J.Pharmac. 52, 110-111p

Horton,E.W. & Main,I.H.M.(1963) Br.J.Pharmac. 21, 182-189

Horton,E.W. & Main,I.H.M.(1965) Br.J.Pharmac.Chemother. 24,470-6

Horton,E.W. & Main,I.H.M.(1967) Br.J.Pharmac. 30, 568-581

Horton,E.W.(1963) Nature (Lond.) 200, 892

Horton,E.W.(1972) IN: Prostaglandins, Monographs on Endocrinology, vol.7 ed. Gross,F., Labhart,A., Mann,T., Samuels,L.T. & Zander,J. p31, London, William Heinemann Medical Books Ltd.

Horton,E.W.(1979) Br.Med.Bull. 35, 295-300

Hynie,S., Cepelik,J., Cernohorsky,M., Klenerova,V., Skrivanova,J. & Wenke,M.(1975) Prostaglandins 10, 971

Illes,P. & Knoll,J.(1975) Pharmac.Res.Commun. 7, 37-47

Ishizawa,M. & Minowa,T.(1982) Prostaglandins 24, 843-850

Johansson,C., Aly,A., Nilsson,E. & Flemstrom,G.(1983) In: Advances in Prostaglandin, Thromboxane and Leukotriene Research, vol.12 ed. Samuelsson,B., Paoletti,R . & Ramwell.P. pp395-401 New York, Raven Press

Johnson,M., Jessup,R. & Ramwell,P.W.(1974) Prostaglandins 6, 433-449

Jones,R.L. & Wilson,N,H.(1981) Br.J.Pharmac. 73, 220-221p

Jones,R.L. & Wison,N.H.(1980) In: Advances in Prostaglandin and Thromboxane Research vol.6 ed. Samuelsson,B., Ramwell.P. & Paoletti,R. pp467-483 New York, Raven Press

Jones,R.L.(1970) Ph.D. Thesis. Univ. London.

Jones,R.L.(1976) In: Advances in Prostaglandin and Thromboxane Research, vol.1, ed. Samuelsson,B. & Paoletti,R. pp221-230, New York, Reven Press

Jones,R.L.(1978) Acta Biol.Med.Germ. 37, 837-844

Jones,R.L., Peesapati,V. & Wilson,N.H.(1982) Br.J.Pharmac. 76, 423-438

Jones,R.L., Wilson,N.H. & Marr,C.G.(1979) In: Chemistry. Biochemistry and Pharmacological Activity of Prostanoids, ed. Roberts.S.M. & Scheinmann,F. pp210-220 Oxford, Pergamon Press

Jones,R.L., Wilson,N.H., Amstrong,R.A., Peesapati,V. & Smith.G.M.(1983) In: Advances in Prostaglandin, Thromboxane and Leukotriene Research, vol.11 ed. Samuelsson,B.,Paoletti,R. & Ramwell,P. pp345-350 New York, Raven Press

Joost.H.G., Beckmann,J., Holze,S., Lenzen,S., Posner,W. & Hasselblatt,A.(1976) Diabetologia 12, 201-206

Kadlec,O., Masek,K. & Seferna,I.(1978) J.Pharmac.Exp.Ther. 205, 635-645

Kantor,M.S., Tao,P. & Kiefer,H.C.(1974) Proc.Natl.Acad.Sci.U.S.A. 71, 1317

Karaplis,A.C. & Powell,W.S.(1981a) J.Biol.Chem. 256, 2414-2419

Karaplis,A.C. & Powell,W.S.(1981b) Endocrinology 109, 2124-2128

Karim.S.M.M., Adaikan,P.G. & Kottegoda,S.R.(1980) In: Advances in Prostaglandin and Thromboxane Research vol.7 ed. Samuelsson,B., Ramwell,P.W. & Paolette,R. pp969-980, New York, Raven Press

Kaser-Glanzmann,E., Gerber,E. & Luscher,E.F.(1979) Biochim.Biophys.Acta 558, 344-347

Kaser-Glanzmann,R., Jakabova,M., George,J.N. & Luscher,E.F.(1978) Biochim. Biophys.Acta 512, 1-12

Kenakin,T.P.(1981) J.Pharmac.Exp.Ther. 216, 210-219

Kennedy,I., Coleman,R.A., Humphrey,P.P.A., Levy,G.P. & Lumley,P.(1982) Prostaglandins 24, 667-689

Kimball,F.A. & Wyngarden,L.J.(1975) Prostaglandins 9, 413-429

Kimball,F.A., Kirton,K.T., Spilman,C.H. & Wyngarden,L.J.(1975) Biol.Reprod. 13, 482-489

Kinoshita,F., Nakai,Y., Katakami,H., Imura,H., Shimizu,T. & Hayaishi.O.(1982) Endocrinology 110, 2207-2209

Kohlhardt,M., Bauer,B., Krause,H. & Fleckenstein,A.(1972) Pflugers Arch. 355, 309-322

Kuehl,F.A.Jr. & Humes,J.L.(1972) Proc.Natl.Acad.Sci.U.S.A. 69, 480-484

Kuehl,F.A.Jr., Oien,H.G. & Ham,E.A.(1974) In: Prostaglandin Synthetase Inhibitors ed. Robinson,H.J. & Vane,J.R. p53 New York, Raven Press

Lampugnani,M.G. & de Gaetano,G.(1983) Thromb.Haemol. 50, 451p

Le Breton,G.C. & Dinerstein,R.J.(1977) Thromb.Res. 10, 521-523

Le Breton,G.C. & Feinberg,H.(1974) Pharmacologist 16, 699 (abst.)

Le Breton,G.C., Dinerstein,R.J., Roth,L.J. & Feinberg,H.(1976) Biochem.Biophys.Res.Comm. 71, 362-370

Le Duc,L.E. & Needleman,P.(1979) J.Pharmac.Exp.Ther. 211, 181-188

Lefer.A.M., Smith.E.F.III, Araki,H., Smith,J.B., Aharony,D., Claremon,D.A., Magolda,R.L. & Nicolaou,K.C.(1980) Proc.Natl.Acad.Sci.U.S.A. 77, 1706-1710

Lefkowitz,R.J., Millikin,D., Wood,C.L., Gore.T.B. & Mukherjee,C.(1977) J.Biol.Chem. 252, 5295

Lin,M.T. & Rao,Ch.V.(1978) Life Sci. 22, 303

Lowe,D.A., Matthews,E.K. & Richardson,B.P.(1981) Br.J.Pharmac. 74, 651-663

MacDermot,J., Barnes,P.J., Waddell,K.A., Dollery.C.T. & Blair,I.A.(1981) Eur.J.Pharmac. 75, 127-130

MacDermot.J., Blair,I.A. & Cresp,T.M.(1981) Biochem.Pharmac. 30, 2041-2044

MacIntyre,D.E. & Gordon,J.L.(1977) Thromb.Res. 11, 705-713

MacIntyre,D.E. & Willis,A.L.(1978) Br.J.Pharmac. 63, 316p

MacIntyre,D.E.(1981) In: Platelets in Biology and Pathology. II. ed. Gordon pp211-247 Elsevier, North-Holland Biomedical Press

MacIntyre,D.E., Salzman.E.W. & Gordon,J.L.(1978) Biochem.J. 174, 921-929

Magerlein,B.J., Bundy,G.L., Lincoln,F.H. & Youngdale,G.A.(1975) Prostaglandins 9, 5

Magerlein,B.J., DuCharme,D.W., Magee,W.E., Miller,W.L., Robert,A. & Weeks,J.R.(1973) Prostaglandins 4, 143

Main,I.H.M. & Whittle,B.J.(1975) Br.J.Pharmac. 54, 309-317

Main,I.H.M.(1964) Br.J.Pharmac.Chemother. 22, 511-519

Malagodi,M.H. & Chiou,C.Y.(1974) Eur.J.Pharmac. 27, 25-33

Manzini,S., Maggi,C.A. & Meli,A.(1983) J.Pharm.Pharmac. 35, 584-589

Marrazzi,M.A. & Matschinsky,F.M.(1972) Prostaglandins 1, 373-387

Mathe,A.A., Strandberg,K. & Astrom,A.(1971) Nat.New.Biol. 230, 215

Mathe,A.A., Strandberg,K. & Fredholm,B.(1972) J.Pharm.Pharmac. 24, 378

Mattioli.M., Galeati.G. & Seren,E.(1983) Prostaglandins, Leukotrienes and Medicine 11, 259-268

McDonald,J.W.D. & Stuart,R.R.(1973) J.Lab.Clin.Med. 81, 838

McDonald,J.W.D. & Stuart,R.K.(1974) J.Lab.Clin.Med. 84, 111

McQueen,D.S. & Ungar,A.(1969) In: Prostaglandins, Peptides and Amines, ed. Mantegazza.P. & Horton,E.W., pp123-124, London, Academic Press

Melman,K.L., Weinstein,Y., Sheaver,G.H. & Bourne,H.R.(1974) In: Cyclic AMP Cell Growth and Immune Response ed. Lichtenstein,L.M. & Parker,C.W. p114, New York, Springer-Verlag

Miller,O.V. & Gorman,R.R.(1979) J.Pharmac.Exp.Ther. 210, 734

Miller,O.V. & Magee,W.E.(1973) In: Advances in Biosciences, vol.9 ed. Berstrom,S. & Bernhard,J. pp83-89 Oxford, Pergamon Press

Moncada,S., Gryglewski,R., Bunting,S. & Vane,J.R.(1976a) Nature (Lond.) 263, 663-665

Moncada,S., Gryglewski,R., Bunting,S. & Vane,J.R.(1976b) Prostaglandins 12, 715-737

Moncada,S., Needleman,P., Bunting,S. & Vane,J.R.(1976) Prostaglandins 12, 323-335

Moore,W.V. & Wolff,J.(1973) J.Biol.Chem. 248, 5705

Morton,D.R., Bundy,G.L., Nishizawa,E.E.(1979) In: Prostacyclin, ed. Vane,J.R. & Bergstrom,S. p31 New York, Raven Press

Moskowitz,J., Harwood,J.P., Reid,W.D. & Krishna,G.(1971) Biochim.Biophys.Acta 230, 279

Murphy,R.C., Hammarstrom,S. & Samuelsson,B.(1979) Proc.Natl.Acad.Sci. U.S.A. 76, 4275

Nakano,J., Prancan,A.V. & Moore.S.(1971) Clin.Res. 119, 712

Needleman,P., Minkes,M. & Raz,A.(1976) Science 193, 163

Needleman,P., Moncada,S.. Bunting,S., Vane,J.R., Hamberg,M. & Samuelsson,B.(1976) Nature (Lond.) 261, 558-560

Needleman,P., Raz,A., Minkes.M.S., Ferrendelli.J.A. & Sprecher,H.(1979) Proc.Natl.Acad.Sci.U.S.A. 76, 944

Newcombe,D.S., Ciosek,C.P., Ishikawa,Y. & Fahey.J.V.(1975) Proc.Natl.Acad.Sci.U.S.A. 72, 3124.

Nicolaou,K.C., Barnette,W.E., Gasic,G.P., Magolda,R.L.(1977) J.Am.Chem.Soc. 99, 7736

Nicolaou,K.C., Magolda,R.L., Smith,J.B., Aharony,D., Smith,E.F. & Lefer,A.M.(1979) Proc.Natl.Acad.Sci.U.S.A. 76, 2566-2570

Northover,B.J.(1977) Gen.Pharmac. 8, 293-296

O'Brien,J.R.(1971) In: Thrombosis and Bleeding Disorders, ed. Bang,N.U., Beller,F.K., Deutsch,E. & Mammen,E.F. pp430-436 New York, Georg Thieme Verlag, Stuttgart Academic Press

Oelz,O., Oelz,R., Knapp,H.R., Sweetman,B.J. & Oates,J.A.(1977) Prostaglandins 13, 225-234

Ohuchida,S., Hamanaka,N. & Hayashi,M.(1981) J.Am.Chem.Soc. 103, 4597-4599

Okamici,N. & Terayama,H.(1977) Biochim.Biophys.Acta 465, 54

Omini,C., Brunelli.G., Folco,G.C., Marini,A., Pasargiklian,R. & Berti.F.(1981) Prostaglandins 21, 345

Opmeer,F.A., Adolfs,M.J.P. & Bonta,I.L.(1983) Biochem.Biophys. Res.Commun. 114, 155-161

Park,M.K. & Dyer,D.C.(1973) Prostaglandins 3, 913

Parsons,M. & Sykes,C.(1980) Br.J.Pharmac. 69, 6-7

Paton,W.D.M.(1961) Biochem.Biophys.Res.Commun. 29, 92

Pickles,V.R.(1967) In: Prostaglandins, Nobel Symposium 2, ed. Bergstrom,S. & Samuelsson,B. pp79-83 Stockholm, Almqvist & Wiksell

Pike,J.E., Kupeicki,F.P. & Weeks,J.R.(1967) In: Prostaglandins, Nobel Symposium 2, ed. Bergstrom,S. & Samuelsson,B. pp283-292 Stockholm, Almqvist & Wiksell

Posner,J.(1970) Br.J.Pharmac. 40, 163-164p

Posner,J.(1973) Br.J.Pharmac. 49, 415-427

Powell,W.S., Hammarstrom,S. & Samuelsson,B.(1973) Eur.J.Biochem. 41, 103

Powell,W.S., Hammarstrom,S. & Samuelsson,B.(1974) Eur.J.Biochem. 41, 103-107

Powell,W.S., Hammarstrom,S. & Samuelsson,B.(1975) Eur.J.Biochem. 56, 73-77

Pressman,B.C.(1976) Ann.Rev.Biochem. 45, 501-530

Puurumen,J.(1983) Br.J.Pharmac. 80, 255-262

Rabinowitz,I., Ramwell,P.W. & Davison,P.(1971) Nature(Lond.) 233, 88-89

Ramwell.P.W., Karanian,J.W. & Foegh,M.L.(1982) In: Prostaglandins and Cancer, The First International Conference, ed. Powles,T.J., Bockman,R.S., Honn,K.V. &

Ramwell,P. pp47-65 New York, Alan R Liss Inc.

Rao,Ch.V.(1976) Fed.Proc. 35, 458

Rao,Ch.V.(1977) Life Sci. 20, 2013

Raz,A. & Aharony,D.(1978) Res.Commun.Chem.Pathol.Pharmac. 21, 507-515

Reed,P.W. & Knapp,H.R.(1978) Ann.N.Y.Acad.Sci. 307, 445-447

Remold-O'Donnell,E. (1974) J.Biol.Chem. 249, 3615

Riccioppo Neto,F.(1983) Br.J.Pharmac. 80, 335-341

Richardson,B.P.(1976) Br.J.Pharmac. 56, 356-357p

Riehl,R.M. & Harper,M.J.K.(1981) Endocrinology 108, 18

Rink,T.J., Smith,S.W. & Tsien,R.Y.(1982) FEBS Lett. 148, 21-26

Robison,G.A., Arnold,A. & Hartmann,R.C.(1969) Pharmac.Res.Comm. 1, 325

Rosen,M.R., Ilvento,J.P., Gelband,H., Merker,C.(1974) J.Pharmac.Exp.Ther. 189, 414-422

Roth,D.M., Lefer,A.M., Smith,J.B. & Nicolaou,K.C.(1982) Prostaglandins,Leukotrienes and Medicine 9, 503-509

Rotondi,A. & Knazek,R.A.(1982) Prostaglandins,Leukotrienes and Medicine 9, 45-53

Rucker.W. & Schror.K.(1983) Biochem.Pharmac. 32, 2405-2410

Ruffolo,R.R.Jr., Waddell,J.E. & Yaden,E.L.(1980) J.Pharmac.Exp.Ther. 213, 267-272

Sadovsky,E., Dora,A., Pfeifer,Y., Polishuk,W.Z., Rachaminoff,R. & Sulman,F.G.(1973) Gynecol.Invest. 4, 140-147

Samuelsson,B.(1963) J.Am.Chem.Soc. 85, 1878

Samuelsson.B., Granstrom,E., Green,K. & Hamberg,M.(1971) Ann.N.Y.Acad.Sci. vol.180 ed. Ramwell,P. & Shaw.J. pp138-163

Sanger,G.J. & Bennett,A.(1980) J.Pharm.Pharmac. 32, 705-708

Sanger.G.J. & Bennett,A.(1980a) In: The Prostaglandin System. ed.Velo,G.P. & Berti.F. pp377-391, New York, Plenum Press

Sanner,J.H. & Eakins,K.E.(1976) In: Prostaglandins, Chemical and Biochemical Aspects, ed. Karim,S.M.M. pp139-189 Lancater, MTP Press

Sanner.J.H.(1969) Arch.Int.Pharmacodyn.Ther. 180, 46-56

Sanner,J.H.(1974) Arch.Int.Med. 133, 133-146

Schaaf,T.K., Bindra,J.S., Eggler,J.F., Plattner,J.J., Nelson,A.J., Johnson,M.R., Constantine,J.W., Hess,H-J. & Elger,W.(1981) J.Med.Chem. 24, 1353-1359

Schaaf.T.K., Johnson,M.R., Constantine,J.W., Bindra,J.S., Hess,H-J & Elger,W.(1983) J.Med.Chem. 26, 328-334

Schafer,A.I., Cooper,B., O'Hara,D. & Handin,R.I.(1979) J.Biol.Chem. 254, 2914

Schaumberg,B.P.(1973) Biochim.Biophys.Acta 326, 127

Schillinger,E. & Prior,G.(1980) Biochem.Pharmac. 29, 2297-2299

Schillinger,E., Prior,G., Speckenbach,A. & Wellershoff,S.(1979) Prostaglandins 18, 293-302

Schror,K., Darius,H., Matzky,R. & Ohlendorf,R.(1981) Naunyn-Schmiedebergs Arch.Pharmac. 316, 252-255

Sheard,P.(1968) J.Pharm.Pharmac. 20, 232(letter)

Siegl,A.M., Smith,M.J. & Silver,M.J.(1980) In: Advances in Prostaglandin and Thromboxane Research, vol.6 ed. Samuelsson.B., Ramwell,P.W. & Paoletti,R. New York, Raven Press

Siegl,A.M., Smith,M.J. & Silver,M.J.(1979) Biochem.Biophys.Res.Comm. 90, 291

Siegl,A.M., Smith.M.J., Silver,M.J, Nicolaou,K.C. & Ahern,D.(1979) J.Clin.Invest. 63, 215

Sirois,P., Borgeat,P. & Jeason,A.(1981) J.Pharm.Pharmac. 33, 466-468

Smigel,M. & Fleischer.S.(1974) Biochim.Biophys.Acta 332, 358

Smith.J.B., Ingerman,C.M. & Silver,M.J.(1977) In: Prostaglandins in Hematology ed. Silver,M.J., Smith,J.B. & Kocsis.J.J. New York, Spectrum Publications,Inc.

Smith.J.B., Ingerman,C.M., Kocsis,J.J. & Silver.M.J.(1974) Thromb.Res. 4 (Suppl.1),49

Sorrentino,L., Capasso,F. & Dirosa.M.(1972) Eur.J.Pharmac. 17, 306-308

Spannhake,E.W., Levin,J.L., Hyman,A.L. & Kadowitz,P.J.(1981) Prostaglandins 21, 267

Stinger,R.B., Fitzpatrick,T.M., Corey,E.J., Ramwell,P.W., Rose,J.C. & Kot,P.A.(1982) J.Pharmac.Exp.Ther. 220, 521-525

Stinger,R.B., Fitzpatrick,T.M., van Dam,J., Ramwell,P.W. &

Kot,P.A.(1980) Prostaglandins 19, 213-220

Su,F.Y., Cubeddu,L. & Perkins,J.P.(1976) J.Cyclic Nucleotide.Res. 2, 257

Sun,F.(1977) Biochem.Biophys.Res.Commun. 74, 1432-1440

Svensson,J., Strandberg,K., Tuvemo,T. & Hamberg,M.(1977) Prostaglandins 14, 425

Sweatman,W.J.F. & Collier,H.O.J.(1968) Nature (Lond.) 217, 69

Tateson,J.E., Moncada.S. & Vane,J.R.(1977) Prostaglandins 13, 389-397

Temesvari-Major,E., Gruber,L., Tomoskozi,I., Kovacs,G. & Cseh,G.(1980) Tetrahedron Letters 21, 4035-4038

Tepperman,B.L. & Soper,B.D.(1981b) Prostaglandins 22, 205-212

Tepperman,B.L. & Soper,B.D.(1983) Prostaglandins 25, 425-441

Tepperman,B.L. & Soper,B.D.(1981a) Am.J.Physiol. 241, G313

Tolman,E.L., Partridge,R. & Barris,E.T.(1977) Prostaglandins 14, 11-19

Tuong,C.C., Ferrand,C., Aubert,D., Loubrie,J-C. & Tuong,A.(1982) Biochem.Pharmac. 31, 1147-1150

Tyers,M.B. & Haywood,H.(1979) Agents and Actions (Suppl) 8, 65-78

Ubatuba,F.B.(1973) Br.J.Pharmac. 49, 662-666

Ueno,R., Honda,K., Inoue,S. & Hayaishi,O.(1983) Proc.Natl.Acad.Sci.U.S.A. 80, 1735-1737

Ueno,R., Ishikawa,Y., Nakayama,T. & Hayaishi,O.(1982) Biochem.Biophys.Res.Commun, 109, 576-582

Ueno,R., Narumiya,S., Ogorochi,T., Nakayama,T., Ishikawa,Y. & Hayaishi,O.(1982) Proc.Natl.Acad.Sci.U.S.A. 79, 6093-6097

van Alphen,G.W.H.M. & Angel,M.A.(1975) Prostaglandins 9, 157-166

van Alphen,G.W.H.M., Dutilh,C.E. & de Deckere,E.A.M.(1978) Prostaglandins and Medicine 1, 151-158

Villanueva.R., Hinds,L., Katz,R.L. & Eakins,K.E.(1972) J.Pharmac.Exp.Ther. 180, 78

von Dorp,D.A., Beerthuis,R.K., Nugteren,D.H. & Vonheman,H.(1964) Biochim.Biophys.Acta 90, 204

von Dorp,D.A., Jouvenaz,G.H. & Struijk,C.B.(1967)

Biochim.Biophys.Acta. 137, 396-399

von Euler,U.S.(1934) Naunyn-Schmiedebergs Arch.Exp.Pathol.Pharmakol. 175, 78-84

Vonkeman,H. & von Dorp,D.A.(1968) Biochim.Biophys.Acta 164, 430

Wasserman,M.A., Ducharme,D.W., Wendling,M.G., Griffin,R.L. & Degraaf,G.L.(1980) Eur.J.Pharmac. 66, 53

Weiss,H.J., Willis,A.L., Kuhn,D. & Brand,H.(1976) Br.J.Haematol. 32, 257-271

Welburn,P.J. & Jones,R.L.(1978) Prostaglandins 15, 287-296

White.J.G.(1972) Am.J.Pathol. 66, 295-312

White,J.G., Rao,G.H.R. & Gerrard,J.M.(1974) Am.J.Phathol. 77, 135-150

Whittle,B.J.R.(1981) Gastroenterology 80, 94-98

Whittle,B.J.R., Moncada,S. & Vane,J.R.(1978) Prostaglandins 16, 373-388

Willis,A.L. & Kuhn,D.C.(1974) Prostaglandins 4, 127

Willis,A.L., Vane,F.M., Kuhn,D.C., Scott,C.G. & Petrin,M.(1974) Prostaglandins 8, 453

Wright,K., Luborsky-Moore.J.L. & Berman,H.R.(1979) Molecular Cellular Endocrinol. 13, 25

Wright,K., Pang,C.Y. & Berman,H.R.(1980) Endocrinology 106, 1333

Yagasaki,O., Takai,M. & Yanagiya,I.(1980) Jap.J.Pharmac. 30, 853-860

Zucker,M.B. & Peerschke,E.I.(1980) In: Platelets: Cellular Response Mechanisms and Their Biological Significance.ed. Rotman,A., Meyer,F.A., Gitler,C. & Silberberg,A. John Wiley & Sons Ltd.

Part Two

Adunyah,E.S., Niggli,V. & Carafoli,E.(1982) FEBS Lett. 143, 65-68

Affolter,H., Chiesi,M., Dabrowska,R. & Carafoli.E.(1976) Eur.J.Biochem. 67, 389-96

Akiyama.S.-I., Ono,M. & Kuwano,M.(1983) Biochem.Biophys.Res.Comm. 110, 783-788

Al Timin,K.S., Bedwani,J.R. & Stanton,A.W.B.(1978) Br.J.Pharmac. 63, 167-176

Andersson,K.-E., Edvinsson,L., MacKenzie,E.T., Skarby.T. & Young,A.R.(1983) Br.J.Pharmac. 79, 135-140

Appel,W.(1962) Arzneimittel-Forschung 12, 562-566

Armstrong,J.M., Thirsk,G. & Salmon,J.A.(1979) Hypertension 1, 309-315

Ash,G.R. & Bygrave,F.L.(1977) FEBS Lett. 78, 166-168

Atlas,D. & Adler,M.(1981) Proc.Natl.Acad.Sci.U.S.A. 78, 1237-1241

Baker,P.F. & McNaughton,P.A.(1976) J.Physiol.(Lond.) 259, 103-144

Baker,P.F. & McNaughton,P.A.(1978) J.Physiol.(Lond.) 276, 127-150

Baker,P.F.(1976) Fed.Proc. 35, 2589-2595

Baker,P.F.(1978) Ann.N.Y.Acad.Sci. 307, 250-268

Balzer.H.(1972) Naunyn-Schmiedebergs Arch.Pharmac. 274, 256-272

Barnard,T. & Afzelius,B.A.(1972) Subcell.Biochem. 1, 375-389

Bassingthwaighte,J.B., Fry,C.H. & McGuigan,J.A.S.(1976) J.Physiol. 262, 15-37

Batra,S.(1973) Biochim.Biophys.Acta 305, 428-432

Batra,S.C. & Daniel,E.E.(1971) Comp.Biochem.Physiol. 38A, 369-385

Batra,S.C.(1974) Biochem.Pharmac. 23, 89-101

Bayer,R., Klausche,D., Kaufmann,R. & Mannhold,R.(1975) Arch.Pharmac. 290, 81-97

Bennett,H.S.(1963) J.Histochem.Cytochem. 11, 14-23

Bianchi,C.P. & Bolton,T.C.(1967) J.Pharmac.Exp.Ther. 157, 388-405

Bindoli,A. & Fleischer,S.(1983) Arch.Biochem.Biophys. 221, 458-466

Blackmore,P.F., El-Refai.M.F. & Exton,J.H.(1979) Mol.Pharmac. 15, 598-606

Blaustein,M.P. & Hodgkin,A.L.(1969) J.Physiol.(Lond.) 200, 497-527

Blaustein,M.P. & Russell,J.M.(1975) J.Membr.Biol. 22, 285-312

Blaustein,M.P.(1974) Rev.Physiol.Biochem.Pharmac. 70, 33-82

Blaustein,M.P.(1977) Biophys.J. 20, 79-111

Blaustein,M.P., Ratzlaff,R.W. & Kendrick,N.K.(1978) Ann.N.Y. Acad.Sci. 307, 195-211

Blaustein,M.P., Russell.J.M. & DeWeer.P.(1974) J.Supramol.Struct. 2, 558-581

Blitz,A.L., Fine,R.E. & Toselli,P.A.(1977) J.Cell.Biol. 75, 135-147

Bolton,T.B.(1979) Physiol.Rev. 59, 606-718

Bondi,A.Y.(1978) J.Pharmac.Exp.Ther. 205, 49-57

Bonucci,E., Derenzini,M. & Marinozzi,V.(1973) J.Cell.Biol. 59, 185-211

Borle,A.B.(1968) J.Cell.Biol. 36, 567-582

Borle,A.B.(1973) Fed.Proc. 32, 1944-1950

Bostrom,S-L., Ljung,B., Mardh,S., Forsen,S. & Thulin,E.(1981) Nature (Lond.) 292, 777-778

Boullin,D.J.(1980) In: Cerebral Vasospasm, pp120-124 Chichester, John Wiley & Sons.

Bowman,A. & Gillespie,J.S.(1982) J.Physiol. 328, 11-25

Brandt,L., Andersson,K.E., Edvinsson,L. & Ljunggren,B.(1981) J.Cereb.Blood Flow Metab. 1, 339-347

Brandt,P.W.(1962) Circulation 26, 1075-1091

Brinley,F.J.Jr.(1978) Ann.Rev.Biophys.Bioeng. 7, 363-392

Brinley,F.J.Jr., Tiffert,T. & Scarpa,A.(1978) J.Gen.Physiol. 72, 101-127

Bruns,D.E., McKonald,J.M. & Jarett,L.(1976) J.Biol.Chem. 251, 7191-7197

Burchfield,D.M. & Rall,J.A.(1981) Fed.Proc. 40, 614

Burgos,M.H.(1960) Anat.Rec. 137, 171

Byczkowdki,J.Z.(1973) Arch.Toxic. 31, 137-145

Byczkowski,J.Z. & Borysewicz,R.(1979) Gen.Pharmac. 10, 369-372

Byczkowski,J.Z. & Borysewicz,R.(1976) Gen.Pharmac. 7, 365-369

Byczkowski,J.Z. & Korolkiewicz,Z.E.(1978) Gen.Pharmac. 9, 55-57

Byczkowski,J.Z. & Porter,C.W.(1981) Proceedings of VIth School on Biophysics of Membrane Transport p370, Poland

Byczkowski,J.Z.(1977) Bromat.Chem.Toksykol. 10, 413-430

Byczkowski.J.Z.(1978) Gen.Pharmac. 9, 425-430

Byczkowski,J.Z.(1983) Gen.Pharmac. 14, 413-417

Byczkowski,J.Z., Salamon,W., Harlos,J.P. & Porter,C.W.(1981) Biochem.Pharmac. 30, 2851-2860

Byczkowski,J.Z., Zychinski,L. & Porter,C.W.(1982) Biochem.Pharmac. 31, 4045-4053

Bygrave,F.L.(1967) Nature (Lond.) 214, 667-671

Bygrave,F.L.(1978) Biol.Rev. 53, 43-79

Carafoli,E. & Azzi,A.(1972) Experientia 27, 906-908

Carafoli,E. & Crompton,M.(1978b) Curr.Top.Membr.Transp. 10, 151-216

Carafoli,E. & Crompton,M.(1978a) Ann.N.Y.Acad.Sci. 307, 269-284

Carafoli.E. & Tiozzo,R.(1967) Experientia 23, 1017-1020

Carafoli,E., Niggli,V., Malmstrom,K. & Caroni,P.(1980) Ann.N.Y.Acad.Sci. 356, 258-266

Carafoli,E., Tiozzo,R., Rossi,C.S. & Lugli,G.(1972) In: Role of membranes in secretory processes, ed. Bolis,L, Keynes,R.D. & Wilbrandt,W. pp175-181 Amsterdam, North Holland

Carey,M.C., Hirom,P.C. & Small,D.M.(1976) Biochem.J. 153, 519-531

Caroni,P. & Carafoli,E.(1981) J.Biol.Chem. 256, 3263-3270

Carsten,M.E.(1973) Am.J.Obstet.Gynecol. 117, 824-832

Carvalho,A.P., Sanui,H. & Pace,N.(1963) J.Cell.Physiol. 62, 311-317

Casteels,R. & Login,I.S.(1983) J.Physiol.(Lond.) 340, 403-414

Casteels,R.(1980) Chest 78 (Suppl.),150-156

Casteels,R., Goffin,J., Raeymaekers,L. & Wuytack,F.(1973) J.Physiol.(Lond.) 231, 19p

Chambers,R.(1940) Cold Spring Harbor Symp.Quant.Biol. 8, 144-153

Chance,B.(1965) J.Biol.Chem. 240, 2729-2748

Chazotte,B. & Vanderkooi,G.(1981) Biochem.Biophys.Acta 636, 153-161

Chefurka,w., Zahradka,P. & Bajura,S.T.(1980) Biochim.Biophys.Acta 601, 349-357

Cheung,W.Y.(1980) Science 207, 19-27

Cheung,W.Y.(1980, 1982, 1983) ed. Calcium Cell Funct. vol.1, 2, 3

Claret-Berthon,B., Claret,M. & Mazet,J.L.(1977) J.Physiol.(Lond.) 272, 529-552

Coates,J. & Weetman,D.F.(1983) Br.J.Pharmac. 80, 343-346

Coates.J., Jahn,U. & Weetman,D.F.(1982) Br.J.Pharmac. 75, 549-552

Coceani,F. & Wolfe,L.S.(1966) Can.J.Physiol.Pharmac. 44, 933-950

Cook,G.M.W., Laico,M.T. & Eylar,E.H.(1965) Proc.Natl.Acad.Sci.U.S.A. 54, 247-252

Daniel,E.E., Crankshaw,D.J. & Kwan,C.-Y.(1979) In: Trends in autonomic Pharmacogy, vol.1 ed. Kalsner,S. pp443-484 Baltimore, Urban and Schwarzenberg

De Jonge,A., Mathy,M.J., Thoolen,M.J.M.C., Timmermans,P.B.M.W.M., Wilffert,B. & van Zwieten,P.A.(1983) Br.J.Pharmac. 80 (Suppl.),480p

Deth,R. & van Breemen,C.(1974) Pflugers Arch. 348, 13-22

Deth,R. & van Breemen,C.(1977) J.Membr.Biol. 30, 363-380

DiPolo,R.(1976) Fed.Proc. 35, 2579-2582

DiPolo,R.(1979) J.Gen.Physiol. 73, 91-113

Dipolo,R.(1974) J.Gen.Physiol. 64, 603-517

Duffy,M.J. & Schwarz,V.(1973) Biochim.Biophys.Acta 330, 294-301

Duncan,C.J.(1976) J.Neurochem. 27, 1277-1279

Echlin,F.(1971) J.Neurosurg. 35, 646-656

Elias,E. & Boyer,J.L.(1979) Science 206, 1404-1406

Epstein,P.M., Fiss,K., Hachisu,R. & Andrenyak,D.M.(1982) Biochem.Biophys.Res.Comm. 105, 1142-1149

Erman,A., Azuri,R. & Raz,A.(1983) Res.Comm.Chem.Pathol.Pharmac. 40, 465-474

Fairhurst,A.S., Whittakar,M.L. & Ehlert,F.J.(1980) Biochem.Pharmac. 29, 155-162

Farber,J.L., El-Mofty,S.K., Schanne,F.A.X., Aleo,J.J.Jr. & Serroni,A.(1977) Arch.Biochem.Biophys. 178, 617-624

Farley,J.M. & Miles,P.R.(1978) J.Pharmac.Exp.Ther. 207, 340-346

Fleckenstein,A.(1964) Verh.Deutsch.Ges-Inn.Med. 70, 81

Fleckenstein,A.(1977) Ann .Rev.Pharmac.Toxicol. 17, 149-166

Folkert,V.W. & Schlondorff,D.(1983) In: Advances in Prostaglandin, Thromboxane, and Leukotriene Research, vol.11 ed. Samuelsson.B., Paoletti,R. & Ramwell,P. pp513-516 New York, Raven Press

Fournier,N., Crevat,A., Ducet,G. & Murisasco,A.(1983) Biochem.Biophys.Res.Comm. 111, 326-332

Frank,G.B.(1962) J.Physiol.(Lond.) 163, 254-268

French,A.M. & Scott,N.C.(1981) Br.J.Pharmac. 73, 321-323

Fukuda,J. & Kawa,K.(1977) Science 196, 309-311

Gasic,G. & Gasic,T.(1962) Nature (Lond.) 196, 170

Ghijsen,W.E.J.M. & van Os,C.H.(1979) Nature (Lond.) 279, 802-803

Gibbins,I.L. & Haller,C.J.(1979) Cell.Tissue.Res. 200, 257-271

Gillespie,J.S. & Maxwell,J.D.(1971) J.Histochem.Cytochem. 19, 676-681

Gillespie,J.S. & McGrath.J.C.(1973) J.Physiol.(London) 230, 659-672

Gillespie,J.S.(1971) Br.J.Pharmac. 43, 430P

Gillespie,J.S.(1972) Br.J.Pharmac. 45, 404-416

Glossman,H. & Hornung,R.(1980) Mol.Cell.Endocrinol. 19, 243-251

Glossman,H., Ferry,D.R., Lubbecke,F., Mewes,R. & Hoffmann,F.(1982) Trends Pharmac.Sci. p431-437

Gmaj,P., Murer,H. & Kinne,R.(1979) Biochem.J. 178, 549-557

Godfraind,T., Miller,R.C. & Socrates Lima,J.(1983) Br.J.Pharmac. 80, 115-121

Gomez-Puyou,A., Tuena de Gomez-Puyou,M., Becker,G. & Lehninger,A.L.(1972) Biochem.Biophys.Res.Comm. 47, 814-819

Gopinath,R.M. & Vincenzi,F.F.(1977) Biochem.Biophys.Res.Comm. 77, 1203-1209

Gould,R.J., Murphy,K.M.M., Reynolds,I.J., Snyder,S.H.(1983) Proc.Natl.Acad.Sci.U.S.A. 80, 5122-5125

Greenawalt,J.W., Rossi,C.S. & Lehninger,A.L.(1964) J.Cell.Biol. 23, 21-38

Greenberg,S., Kadowitz,P.J., Diecke,P.P.J. & Long,J.P.(1973) Arch.Int.Pharmacodyn.Ther. 205, 318

Grosset,A. & Mironneau,J.(1977) J.Physiol.(Lond.) 270, 765-784

Hagiwara,S.(1975) In: Dynamic Properties of Lipid Membranes and Bilayers,ed. Eisenman,G. pp359-381 New York, Dekker

Harbon,S., Vesin,M.-F. & Do Khac,L.(1975) Colloq.INSERM 50, 83-100

Harigaya,S. & Schwartz,A.(1969) Circ.Res. 25, 781-794

Harris,A., Swamy,V.C., Triggle,D.J. & Waters,D.H.(1980) J.Autonom.Pharmac. 1, 61-66

Harris,E.S. & Baum,H.(1980) Biochem.J. 106, 725-732

Hashimoto,K., Nakagawa,Y., Nabata,H. & Imai,S.(1978) Arch.Int. Pharmacodyn.Ther. 231, 212-221

Hashimoto,K., Takeda,K., Katano,Y., Nakagawa,Y., Tsukada,T., Hashimoto,T., Shimamoto,N., Sakai,K., Otorrii,T. & Imai,S.(1979) Arzneim.Forsch. 29, 1368-1373

Hay,D.W.P. & Wadsworth,R.M.(1983) Eur.J.Pharmac. 87, 367-378

Hay,D.W.P. & Wadsworth,R.M.(1980) Br.J.Pharmac. 68, 182p

Hay,D.W.P. & Wadsworth,R.M.(1982b) Br.J.Pharmac. 76, 103-113

Hay,D.W.P. & Wadsworth,R.M.(1981) Br.J.Pharmac. 72, 563p

Hay,D.W.P. & Wadsworth,R.M.(1982a) Eur.J.Pharmac. 77, 221-228

Heaslip,R.J. & Rahwan,R.G.(1982) Can.J.Physiol.Pharmac. 60, 743-746

Hedqvist,P.(1977) Ann .Rev.Pharmac.Toxicol. 17, 259-279

Hinds,T.R., Larsen,F.L. & Vincenzi,F.F.(1978) Biochem.Biophys. Res.Comm. 81, 455-461

Hirata,M., Suematsu,E. & Koga,T.(1983) Mol.Pharmac. 23, 78-85

Hirata,M., Suematsu,E. & Koga,T.(1982) Biochem.Biophys.Res.Comm. 105, 1176-1181

Ho,M.-M., Scales,D.J. & Inesi,G.(1983) Biochim. Biophys. Acta 730, 64-70

Howell,S.L. & Tyhurst,M.(1976) J.Cell.Sci. 21, 415-422

Hudgins,P. & Weiss,G.B.(1968) J.Pharmac.Exp.Ther. 159, 91-97

Hudgins,P.(1969) J.Pharmac.Exp.Ther. 170, 303-310

Humphrey,P.P.A. & Robertson,M.J.(1983) Br.J.Pharmac. 80 (Suppl.),502p

Imai,S.(1979) Trends Pharmac.Sci. 2, 87-89

Janis,R.A., Crankshaw,D.J. & Daniel,E.E.(1977) Am.J.Physiol. 232, C50-58

Jarrett,H.W. & Penniston,J.T.(1977) Biochem.Biophys.Res.Comm. 77, 1210-1216

Jetley,M. & Weston,A.H.(1980) Br.J.Pharmac. 68, 311-320

Jim,K.(1980) Ph.D. Thesis Buffalo, State University of New York

Johnson,J.D., Vaghy,P.L., Crouch,T.H., Potter,J.D. & Schwartz,A. (1982) In: Advances in Pharmacology and Therapeutics II. Proceedings of the 8th Int.Congress of Pharmacology Tokyo, vol.3 ppl21-138

Kanamori,M., Naka,M., Asano,M. & Hidaka,H.(1981) J.Pharmac.Exp.Ther, 217, 494-499

Kanno,K. & Sasaki,Y.(1982) Biochem.Pharmac. 31, 2977-2981

Karaki,H., Murakami,K., Nakagawa,H., Ozaki,H. & Urakawa,N.(1982) Br.J.Pharmac. 77, 661-666

Kass,R.S. & Tsien,R.W.(1975) J.Gen.Physiol. 66, 169-192

Katz,S. & Remtulla,M.A.(1978) Biochem.Biohys.Res.Comm. 83,

1373-1379

Kawamura,M. & Inagami,T.(1983) Endocrinology 112, 1857-1859

Keefe,D.L. & Kates,R.E.(1982) J.Pharmac.Exp.Ther. 220, 91

Kimura,T., Chu,J.W., Mukai,R., Ishizuka,I. & Ymamkawa,T.(1972) Biochem.Biophys.Res.Comm. 49, 1678-1683

Kinne-Saffran,E. & Kinne,R.(1974) J.Membr.Biol. 17, 263-274

Kirtland,S.J. & Baum,H.(1972) Nature (New Biol.) 236, 47-49

Kohlhardt,M., Bauer,B., Krause,H. & Fleckenstein,A.(1972) Pflugers Arch. 335, 309-322

Koketsu,K., Kitamura,R. & Tanaka,R.(1964) Am.J.Physiol. 207, 509-512

Kretsinger,R.H.(1971) Int.Rev.Cytol. 46, 323

Kretsinger,R.H.(1976) Coord.Chem.Rev. 18, 29

Kurebayashi,N, & Ogawa,Y.(1981) Jpn.J.Pharmac. 31 (Suppl.),214p

Kurebe,M.(1978) Mol.Pharmac. 14, 138-144

Kurebe,M.(1979) Life Sci. 24, 275-282

LePeuch,C.J., Haiech,J. & Demaille,J.G.(1979) Biochemistry 18, 5150-5157

Lehninger,A.L.(1964) The Mitochondrion, New York, Benjamin

Lehninger,A.L.(1970) Biochem.J. 119, 129-138

Lehninger,A.L.(1974) Circ.Res. 35 (Suppl.III),83-90

Lehninger,A.L., Reynafarje,B., Vercesi,A. & Tew,W.P.(1978) Ann.N.Y.Acad.Sci. 307, 160-176

Lentz,R.W., Harrison,C.E.Jr., Dewey,J.D., Barnhorst,D.A., Danielson,G.K. & Pluth,J.R.(1978) J.Mol.Cell.Cardiol. 10, 3-30

Levin,R.M. & Weiss,B.(1977) Mol.Pharmac. 13, 690-697

Loutzenhiser,R.D. & van Breemen,C.(1981) Fed.Proc. 40, 624

Lullmann,H., Timmermans,P.B.M.W.M. & Ziegler,A.(1979) Eur.J. Pharmac. 60, 277

Luttgau,H.C. & Niedergerke,R.(1958) J.Physiol.(Lond.) 143, 486-505

Luttgau,H.C.(1963) J.Physiol.(Lond.) 168, 679-697

Malik,K.U., Ryan,P. & McGiff,J.C.(1976) Circulation Res. 39,

163-169

Malik,K.V.(1978) Fed.Proc. 37, 203-207

Malmstrom,K. & Carafoli,E.(1975) Arch.Biochem.Biophys. 171, 418-423

Malmstrom,K. & Carafoli,E.(1977) Arch.Biochem.Biophys. 182, 657-666

Martin,J.H. & Matthews,J.L.(1970) Clin.Orthop. 68, 273-278

Mas-Oliva,J. & Nayler,W.G.(1980) Br.J.Pharmac. 70, 617-624

Massingham,R.(1973) Eur.J.Pharmac. 22, 75-82

Matsushima,Y. & Gemba,M.(1982) J.Pharmac.Exp.Ther. 222, 452-457

Matthews,J.L., Martin,J.H., Sampson,H.W., Kunin,A.S. & Roan,J.H.(1970) Calcif. Tissue Res. 5, 91-99

McNamara,D.B., Roulet,M-J., Gruetter,C.A., Hyman,A.L. & Kadowitz,P.J.(1980) Prostaglandins 20, 311-320

Meech,R.W. & Standen,N.B.(1975) J.Physiol. (Lond.) 249, 211-239

Meissner,A. & Fleischer,S.(1971) Biochim.Biophys.Acta 241, 356-378

Mela,L.(1977) In: Current topic in membranes and transport, vol.9 ed. Bronner,F. & Kleinzeller,A. pp321-366 New York, Academic Press

Mikkelsen,E., Addersson,K-E. & Lederballe Pedersen,O.(1979) Acta Pharmac.Toxicol. 44, 110-119

Mikkelsen,R.B.(1976) In: Biological Membranes, vol.3 ed. Chapman,D. & Wallach,D.F.H. pp133-190 London, Academic

Moore,L., Fitzpatrick,D.F., Chen,T.S. & Landon,E.J.(1974) Biochim.Biophys.Acta 345, 405-418

Moriaty,C.M.(1977) Mol.Cell.Endocrinol. 6, 349-361

Motlib,M.A., Vaghy,P.L., Johnson.J.D. & Schwartz,A.(1982) Fed.Proc. 40, 1469

Muller-Schweinitzer,E.(1983) Naunyn-Schmiedebergs Arch.Pharmac. 324, 64-69

Naccache,P.H., Molski,T.F.P., Alobaidi,T., Becker,E.L., Showell,H.J. & Sha'afi,R.I.(1980) Biochem.Biophys.Res.Comm. 97, 62-68

Nachsen,D.A. & Blaustein,M.P.(1979) Mol.Pharmac. 16, 579-586

Narayanan,N., Lee,P., Newland,M. & Khandelwal,R.L.(1983)

Biochem.Biophys.Res.Comm. 108, 1158-1164

Nayler,W.G. & Szeto,J.(1972) Cardiovasc.Res. 6, 120-128

Nayler,W.G., Mas-Oliva,J. & Williams,A.J.(1979) In: Recent Advances in Studies on Cardiac Structure and Metabolism, vol.12 ed. Dhalla,N. & Tajuddin,M. Baltimore, University Park Press

Nayler,W.G., Poole-Wilson,P.A. & Williams,A.(1979) J.Mol.Cell. Cardiol. 11, 683-706

Neri Serneri,G.G., Masotti,G., Poggesi,L. & Galanti,G.(1980) Thromb.Res. 17, 197-208

Nguyen Duong,H. & Brecht,K.(1977) Munch.Med.Wochenschr. 119 (Suppl.1),1-S12-18

Niggli,V., Adunyah,E.S. & Carafoli,E.(1981) J.Biol.Chem. 256, 8588-8592

Niggli,V., Adunyah,E.S., Penniston,J.T. & Carafoli,E.(1981) J.Biol.Chem. 256, 395-401

Niggli,V., Penniston,J.T. & Carafoli,E.(1979) J.Biol.Chem. 254, 9955-9958

Nishikawa,M., Tanaka,T. & Hidaka,H.(1980) Nature (Lond.) 287, 853-864

Nishimura,M., Asai,F. & Urakawa,N.(1982) Jpn.J.Pharmac. 32, 231-235

Ohashi.T., Uchida,S., Nagai.K. & Yoshida,H.(1970) J.Biochem.(Tokyo) 67, 635-641

Oriowo,M.A.(1982) Arch.Int.Pharmacodyn. 259, 186-193

Osaka,K.(1977) J.Neurosurg. 47, 403-411

Palmer,P.F. & Posey,V.A.(1970) J.Gen.Physiol. 55, 89-103

Pang,D.C. & Sperelakis,N.(1983) Eur.J.Pharmac. 87, 199-207

Parkinson,D.K. & Radde,I.C.(1971) Biochim.Biophys.Acta 242, 238-246

Parod,R.J. & Putney,J.W.Jr.(1980) Am.J.Physiol. 239, G106-113

Paton,D.M. & Daniel,E.E.(1967) Can.J.Physiol.Pharmac. 45, 795-804

Patriarca,P. & Carafoli,E.(1968) J.Cell.Physiol. 72, 29-38

Peachey,L.D.(1964) J.Cell.Biol. 20, 95-108

Pershadsingh,H.A., Landt,M. & McDonald,J.M.(1980) J.Biol.Chem. 255, 8983-8986

Piascik,M.T., Johnson,C.L., Potter,J.D. & Rahwan,R.G.(1981) Fed.Proc. 40, 1740

Publicover,S.J. & Ducan,C.J.(1979) Eur.J.Pharmac. 54, 119-127

Putney,J.W. & Bianchi,C.P.(1974) J.Pharmac.Exp.Ther. 189, 202-212

Putney,J.W.Jr.(1978) Pharmac.Rev. 30, 209-245

Quinn,P.J. & Dawson,R.M.C.(1972) Chem.Phys.Lipids 8, 1-9

Quirion,R. & Pert,C.B.(1982) Eur.J.Pharmac. 83, 155-156

Quist,E.E. & Roufogalis,B.D.(1977) J.Supramol.Struct. 6, 375-381

Quist,E.E. & Roufogalis,B.D.(1975) Arch.Biochem.Biophys. 168, 240-251

Raess,B.U. & Vincenzi,F.F.(1980) Mol.Pharmac. 18, 253-258

Rahamimoff,H. & Abramovitz,E.(1978) FEBS Lett. 92, 163-167

Rahwan,R.G. & Gerald,M.C.(1981) Can.J.Physiol.Pharmac. 59, 617-620

Rahwan,R.G. & Witiak,D.T.(1979) In: Trace Metals in Health and Disease, ed. Kharasch,N., pp217-225 New York, Raven Press

Rahwan,R.G., Faust,M.M. & Witiak,D.T.(1977) J.Pharmac.Exp.Ther. 201, 126-137

Rahwan,R.G., Witiak,D.T. & Muir,W.W.(1981) Annu.Rep.Med.Chem. 16, 257-268

Rao,C.V. & Mitra,S.B.(1982) Methods in Enzymology 86, 192-202

Reimer,J., Dorfler,F., Mayer,C-J. & Ulbrecht,G.(1974) Pflugers Arch. 351, 241-258

Reiner,O. & Marshall,J.M.(1976) Naunyn-Schmiedebergs Arch.Pharmac. 292, 243-250

Reuter,H. & Seitz,N.(1968) J.Physiol.(Lond.) 195, 451-470

Reynafarje,B., Lehninger,A.L.(1969) J.Biol.Chem. 244, 584-593

Rokutanda,M., Araki,S. & Sakanashi,M.(1983) Arch.Int.Pharmac. 262, 99-108

Rose,B. & Lowenstein,W.R.(1975) Science 190, 1204-1206

Rosenberger.L.B., Ticku,M.K. & Triggle,D.J.(1979)

Can.J.Physiol. Pharmac. 57, 333-347

Ruigrok,J.C. & Elbers,P.F.(1972) Cytobiologie 5, 51-64

Sayegh,F.S., Davis,R.W. & Solomon,G.C.(1974) J.Dent.Res. 53, 581-587

Scarpa,A. & Graziotti,P.(1973) J.Gen.Physiol. 62, 756-772

Scarpa,A.(1975) In: Calcium transport in contraction and secretion, ed. Carafoli,E., Cleminti,F., Drabikowski,W. & Magreth,A. pp65-76 Amsterdam, North Holland

Scarpa,A., Brinley,F.J., Tiffert,T. & Dubyak,G.R.(1978) Ann.N.Y.Acad.Sci. 307. 86-112

Scharf,O. & Foder,B.(1978) Biochim.Biophys.Acta 509, 67-77

Schatzmann,H.J. & Rossi,G.L.(1971) Biochim.Biophys.Acta 241, 379-392

Schatzmann,H.J. & Vincenzi,F.J.(1969) J.Physiol.(Lond.) 201, 369-395

Schatzmann,H.J.(1975) In: Current topics in membranes and transport, vol.6 ed. Bronner,F. & Kleinzeller,A. pp125-168

Schenolikar,S., Cohen,P.T.W., Cohen,P., Nairn,A.C. & Perry,S.V.(1979) Eur.J.Biochem. 100

Schumann,J.H., Gorlitz,B.D. & Wagner,J.(1975) Naunyn-Schmiedeberg Arch.Pharmac. 289, 409-418

Shenolikar,S., Cohen,P.T.W., Nairn,A.C. & Perry,S.V.(1979) Eur.J.Biochem. 100, 329-337

Shigenobu,K., Schneider,J.A. & Sperelakis,N.(1974) J.Pharmac. Exp.Ther. 190, 280-288

Shimizu,K., Ohta,T. & Toda,N.(1980) Stroke 11, 261-266

Simonsen,L. & Christoffersen,G.R.J.(1979) Comp.Biochem.Physiol.(A) 63, 615-618

Solaro,R.J.(1972) Doctoral Thesis, Univ. Pittsburgh School of Medicine

Somlyo,A.P., Somlyo,A.V., Devine,C.E., Peters,P.D. & Hall,T.A.(1974) J.Cell.Biol. 61, 723

Sottocasa,G.L., Sandri,G., Panfili,E., de Bernard,B., Gazzotti,P., Vasington,F.D. & Carafoli,E.(1972) Biochem.Biophys.Res.Comm. 47, 808-813

Sottocasa,G.L., Sandri,G., Panfili,E. & de Bernard,B.(1971) FEBS Lett. 17, 100-105

Sparrow,M.P., Mrwa,U., Hofmann,F. & Ruegg,J.C.(1981) FEBS Lett. 125, 141-145

Spedding,M.(1982) Naunyn-Schmiedebergs Arch.Pharmac. 318, 234-240

Stieger,J. & Schatzmann,H.J.(1981) Cell Calcium 2, 601-616

Stjarne,L.(1978) Acta Physiol.Scand. 102, 251-253

Sulakhe,P.V., Drummond,G.I. & Ng,D.C.(1973) J.Biol.Chem. 248, 4150-4157

Sutfin,L.V., Holtrop,M.E. & Oglivie,R.E.(1971) Science 174, 947-949

Szent-Gyorgyi,A.(1960) In: Introduction to a Submolecular Biology, New York, Academic Press

Tanishima.T.(1980) J.Neurosurg. 53, 787-793

Tanishima,T.(1983) Brain Nerve 35, 323-329

Tashmukhamedov,B.A., Gagelgans,A.I., Mamatkulov,K. & Makhmodova,E.M.(1972) FEBS Lett. 28, 239-242

Thorens,S.(1979) FEBS Lett. 98, 177-180

Tobias,J.M., Agin,D.P. & Pawlowski,R.(1962) Circulation 26, 1145-1150

Triggle,D.J. & Swamy,V.C.(1980) Chest 78 (Suppl.),174-180

Tsokos,J., Sans,R. & Bloom,S.(1977) Life Sci. 20, 1913-1922

Tsuyoshi,S. & Develin,T.M.(1979) Biochem.Biophys.Res.Comm. 89, 240

Tupper,J.T. & Zorgniotti,F.(1977) J.Cell.Biol. 75, 12-22

Vale,M.G.P., Moreno,A.J.M. & Carvalho,A.P.(1983) Biochem.J. 214, 929-935

Vallieres,J., Scarpa,A., Somlyo,A.P.(1975) Arch.Biochem.Biophys. 170, 659-669

van Breeman,C.(1969) Arch.Int.Physiol.Biochim. 77, 710-716

van Breemen,C., Aaronson,P., Loutzenhiser,R. & Meisheri,K.(1980) Chest 78 (Suppl.),157-165

van Breemen,C., Farinas,B.R., Gerba,P. & McNaughton,E.D.(1972) Circulation Res. 30, 44-54

van Rossum,G.D.V.(1970) J.Gen.Physiol. 55, 18-32

van Zwieten,P.A., van Meel,J.C.A. & Timmermans,P.B.M.W.M.(1983) Hypertension 5 (Suppl.II), 8-17

Vincenzi,F.F.(1971) In: Cellular mechanisms for calcium transfer and homeostasis,ed. Nichols,G.Jr. & Wasserman,R.H.

pp135 New York, Academic Press

Vincenzi,F.F., Adunyah,E.S., Niggli,V. & Carafoli,E.(1982) Cell Calcium 3, 545-559

Volpi,M., Sha'afi,R.I., Epstien,P.M., Andrenyak,D.M. & Feinstein,M.B.(1981) Proc.Natl.Acad.Sci.U.S.A. 78, 795-799

Waisman,D.M., Gimble,J.M., Goodman,D.B.P. & Rasmussen,H.(1981) J.Biol.Chem. 256, 409-414

Watanabe,A.M. & Besch,H.R.Jr.(1974) J.Pharmac.Exp.Ther. 191, 241-251

Weibel,E.R., Staubli,W., Gnagi,H.R. & Hess,F.A.(1969) J.Cell.Biol. 42, 68-91

Weinbach,E.C. & von Brand,T.(1965) Biochem.Biophys.Res.Comm. 19, 133-137

Weiner,M.L. & Lee,K.S.(1972) J.Gen.Physiol. 59, 462-475

Weiss,B., Prozialeck,W., Cimino,M., Barnette,M.S., Wallace,T.L.(1980) Ann.N.Y.Acad.Sci. 356, 319-345

Wellum,G.R., Irvine,T.W. & Zervas,N.T.(1980) J.Neurosurg. 53, 486-490

Wikstrom,M.K.F., Ahonen,P. & Luukkainen,T.(1975) FEBS Lett. 56, 120-123

Winkle,W.B.(1976) Science 193, 1130-1131

Zadunaisky,J.A., Gennaro,J.F.Jr., Bashirelahi,N. & Hilton,M.(1968) J.Gen.Physiol. 51, 290-302